Down-Regulation of the Monocarboxylate Transporter 1 Is Involved in Butyrate Deficiency During Intestinal Inflammation

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Background & Aims: **Butyrate oxidation is impaired in intestinal mucosa of patients with inflammatory bowel diseases (IBD). Butyrate uptake by colonocytes involves the monocarboxylate transporter (MCT) 1. We aimed to investigate the role of MCT1 in butyrate oxidation deficiency during colonic inflammation.** ***Methods:*** **Colonic tissues were collected from patients with IBD or healthy controls and from rats with dextran sulfate sodium (DSS)-induced colitis. The intestinal epithelial cell line HT-29 was treated with interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). MCT1 expression was analyzed by real-time reverse-transcription polymerase chain reaction, Western blot, and immunofluorescence. Butyrate uptake and oxidation in HT-29 cells was assessed using [^{14}C]-butyrate. The mechanism of MCT1 gene regulation was analyzed by nuclear run-on and reporter gene assays.** ***Results:*** **MCT1 messenger RNA (mRNA) and protein levels were markedly decreased in inflamed colonic mucosa of IBD patients and rats. In HT-29 cells, down-regulation of MCT1 mRNA and protein abundance by IFN-γ and TNF-α correlated with a decrease in butyrate uptake and subsequent oxidation. IFN-γ and TNF-α did not affect MCT1 mRNA stability but rather down-regulated gene transcription. We demonstrate that the cytokine response element is located in the proximal −111/+213 core region of the MCT1 promoter.** ***Conclusions:*** **The data suggest that butyrate oxidation deficiency in intestinal inflammation is a consequence of reduction in MCT1-mediated butyrate uptake. This reinforces the proposition that butyrate oxidation deficiency in IBD is not a primary defect.**

BASIC–ALIMENTARY TRACT

Butyrate is a short-chain fatty acid produced by colonic bacterial fermentation of dietary fibers. Butyrate plays an important part in maintaining the health and integrity of the colonic mucosa. It is the primary energy source for the colonic epithelium[1] and regulates cell proliferation,[2] differentiation, and apoptosis.[3] In addition, butyrate has an important role in modulating mucosal inflammation.[4,5]

Ulcerative colitis (UC) and Crohn's disease (CD) are the 2 major inflammatory bowel diseases (IBD). Several studies have found that butyrate oxidation is decreased in the inflamed mucosa of patients suffering from UC[6,7] or CD[8] and in animal models of experimental colitis.[9] However, these studies have suggested that butyrate oxidation deficiency is not a primary defect. Indeed, butyrate oxidation is impaired in patients with active UC but not with quiescent UC.[7] Also, in mice with dextran sulfate sodium (DSS)-induced colitis, butyrate oxidation was impaired only after 6 days of DSS treatment.[9] These studies did not consider a potential reduction in butyrate uptake by intestinal epithelial cells as a causative factor of this butyrate oxidation deficiency.

The human monocarboxylate transporter 1 (MCT1) gene encodes for a plasma membrane protein of 45 kilodaltons containing 12 α-helical transmembrane domains with C- and N-termini located within the cytoplasm.[10] We and others have demonstrated that MCT1 transports butyrate across the apical membrane of human colonocytes.[11,12] Thus, a decrease in MCT1 expression, which reduces the intracellular availability of butyrate,[13] could affect not only its oxidation but also its cell regulatory effects. Indeed, we have shown that silencing MCT1 expression by RNA interference in colonic epithelial cells decreases butyrate induction of cell-cycle arrest and differentiation.[13] This finding suggests that a decrease in MCT1 expression could negatively affect colonic tissue homeostasis.

In human colonic tissues, during transition from normality to malignancy, decreased MCT1 expression has been reported.[14] However, very little is known about

Abbreviations used in this paper: CAPE, caffeic acid phenylethyl ester; DSS, dextran sulfate sodium; IFN, interferon; MCT, monocarboxylate transporter; MTT, [4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor.

0016-5085/07/$32.00
doi:10.1053/j.gastro.2007.08.041

MCT1 expression in IBD. We hypothesize that the impairment in butyrate oxidation reported in active IBD could be related to a decrease in MCT1 expression in the inflamed colonic mucosa. We observed that inflammation caused down-regulation of MCT1 expression in the colonic tissue. Furthermore, treatment of intestinal epithelial cell lines with proinflammatory cytokines induced down-regulation of MCT1 expression that was associated with a reduction in butyrate uptake and subsequent oxidation. Therefore, butyrate oxidation deficiency in intestinal inflammation appears to be a consequence of reduction in MCT1-mediated butyrate uptake and sustains the idea that butyrate oxidation deficiency in IBD is not a primary defect.

Materials and Methods

Patients and Biopsies

Colonic biopsy specimens were obtained from inflamed and noninflamed mucosa of 14 patients with CD (9 women, 5 men; mean age, 36 years; range, 21–78 years) and 9 patients with UC (4 women, 5 men; mean age, 46 years; range, 30–62 years). All patients underwent colonoscopy for an active disease. At the time of the study, 9 patients were receiving steroids; 2 patients, 5-aminosalicylic acid; 2 patients, azathioprine; 1 patient, 6-mercaptopurin plus infliximab; 1 patient, cyclosporin; and 8 patients, no medication. Colonic biopsy specimens were collected from healthy mucosa of 10 asymptomatic subjects undergoing routine colonoscopy. The study was approved by the "Fédération des Biothèques" of the University Hospital, Nantes. All patients gave informed consent to take part in the study.

Induction of Colitis in Rats

Principles of laboratory animal care and guidelines according to the Declaration of Helsinki were followed. Sprague-Dawley male rats were treated with 4% DSS in drinking water or with water alone (control) for 5 days. Rats were then killed and colonic segments removed for RNA or protein isolation.

Cell Culture and RNA Interference

The parental intestinal epithelial cell line HT-29 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 2 mmol/L glutamine. Cells were treated with interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) at the indicated time and doses. Cell viability was assessed using the [4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) viability assay (Promega, Charbonnieres, France). To study the effect of IFN-γ and TNF-α on the stability of the MCT1 transcript, cells were stimulated in the presence of 1 μg/mL actinomycin D (Sigma) for 2, 4, 6, 8, and 24 hours. In other experiments, cells were preincubated for 1 hour with 10 μmol/L of the nuclear factor (NF)-κB inhibitor caffeic acid phenylethyl ester (CAPE) (Calbiochem, Nottingham, UK) before treatment with cytokines as described above.

Transfection and Luciferase Assays

pGL3-basic luciferase reporter plasmids containing successive deletions of the 5′-end of the MCT1 promoter (−1525, −1319, −1106, −896, −703, −476, −307, −111) were used to assess promoter activity, as previously described.[15] HT-29 cells were transfected in 96-well plates with 250 ng pGL3-MCT1 plasmid construct or pNFκB-Luc vector (Clontech, Saint-Germain-en-Laye, France) and 100 ng pIRES-EGFP vector (internal control; Clontech) using Lipofectamine 2000 (Invitrogen). Twenty-four hours later, cells were stimulated with increasing doses of TNF-α and IFN-γ for 24 hours. Cell lysates were assayed for luciferase activity using the firefly luciferase 1-step assay kit (Fluoprobes, Montlucon Cedex, France). Luminescence and fluorescence were then measured with a luminometer/fluorimeter (VICTOR[3], PerkinElmer, Courtaboeuf Cedex, France). Luciferase activity was normalized to fluorescence intensity and expressed as relative light units (RLU).

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated with TRIzol reagent (Invitrogen, Cergy Pontoise Cedex, France) and treated for 45 minutes at 37°C with 2 U RQ1 DNAse (Promega). One microgram RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen). One microliter of the complementary DNA (cDNA) solution was subjected to real-time quantitative polymerase chain reaction (PCR) in a Bio-Rad iCycler iQ system using the QuantiTect SYBR Green PCR kit (Qiagen, Courtaboeuf Cedex, France). Quantitative PCR consisted of 45 cycles, each PCR cycle consisting of 30 seconds at 95°C and 30 seconds at 60°C. The sequences of human and rat primers for MCT1, interleukin (IL)-1β, β2-microglobulin, and β-actin are included in Supplementary Table 1 (see Supplementary Table 1 online at www.gastrojournal.org). The expression level of β-actin was used as a reference value to normalize MCT1 and IL-1β gene expression. Relative quantitative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method,[16] using normal tissues from healthy subjects, control rats, or untreated control cells as the calibrator samples.

Western Blot Analysis

Membrane protein samples were either from HT-29 postnuclear membranes, prepared as previously described,[17] or from patient biopsy specimens and rat colonic tissues. The latter were coextracted with RNA using TRIzol reagent.

Samples were analyzed by Western blot using anti-MCT1 antibody (1:1000; Santa Cruz, CA) and a mouse monoclonal anti-β-actin antibody (1:10,000; Sigma, Dorset, UK). Immunoreactive bands were visualized with horseradish peroxidase-conjugated secondary antibody (Dako, Cambridgeshire, UK) and subsequent ECL detection (Amersham International, Buckinghamshire, UK). Band intensities were quantified by scanning densitometry (Phoretix ID quantifier; Non-linear Dynamics, Newcastle, UK).

Immunofluorescence Staining of MCT1

HT-29 cell monolayers were fixed in −20°C methanol, permeabilized, and incubated with the anti-MCT1 antibody or goat IgG control (sc-2028; Santa Cruz) for 1 hour at room temperature, as previously described.[5] This was followed by incubation with FITC-conjugated anti-goat antibody (1:500; Jackson ImmunoResearch, Newmarket, UK). Nuclei were counterstained with Hoechst. Serial x-y and x-z sections were collected every 0.25 μm using a laser scanning confocal microscope (Leica, Rueil-Malmaison Cedex, France). Paraffin-embedded human colonic tissue sections were obtained from the Department of Pathology (Nantes). After microwave treatment in Antigen Unmasking Solution (Vector Laboratories Inc, Peterborough, UK), sections were incubated with the anti-MCT1 antibody (1:100; Santa Cruz) overnight at 4°C, followed by incubation with FITC-conjugated anti-goat antibody. Images were acquired using a digital camera (DXM1200F; Nikon, Champigny sur Marne, France) coupled to a fluorescence microscope (Nikon). Fluorescence intensity was quantified using the LUCIA software (Laboratory Imaging, Champigny sur Marne, France) in 4 regions of interest within the colonic epithelium. Two different tissue sections were analyzed for each patient. Mean fluorescence intensity was calculated from these 8 (4 × 2) values. Image acquisition and analysis were identical between controls (n = 5) and patients (n = 5), allowing a comparative semiquantitative estimation of MCT1 protein expression.

Nuclear Run-On Assays

Run-on assays were performed as described previously.[17] Nuclei were isolated from untreated or cytokine-treated HT-29 cells and incubated in transcription solution (5 mmol/L Tris-HCl [pH 8.0]; 2.5 mmol/L $MgCl_2$; 150 mmol/L KCl; 0.25 mmol/L each of ATP, GTP, and CTP; and 250 μCi [α-^{32}P]UTP [3000 Ci/mmol, 10 μCi/μL]) at 30°C for 20 minutes. The labelled RNA was hybridized to slot-blotted cDNA probes on Hybond-XL nylon membrane and exposed to Biomax-MS film (−80°C for 5 days). Band intensities were assessed densitometrically, and the values were normalized to the β-actin signals.

Uptake and Oxidation of Butyrate

Measurement of cellular uptake and oxidation was performed as previously described,[12,17] with slight modifications. Briefly, HT-29 cells were grown in 24-well plates and treated with IFN-γ and TNF-α as described above. Cells were incubated in Hank's balanced salt solution (HBSS; Invitrogen) containing 10 mmol/L Hepes, pH 7.5, for 30 minutes at 37°C and then in HBSS-10 mmol/L Mes, pH 6.4, containing [^{14}C]-butyrate (16 mCi/mmol) for various incubation times (1–30 minutes). Preliminary kinetic experiments using increasing concentrations of butyrate showed that butyrate uptake was linear between 1 and 10 minutes. Thus, a 5-minute incubation time was used to measure the initial rate of butyrate uptake using butyrate concentrations ranging from 0.1 to 10 mmol/L. K_m and V_{max} values were determined by linear regression using Eadie-Hofstee plots.[17] To assess the effect of cytokines on the steady state rates of butyrate uptake and oxidation, cells were incubated with 40 μmol/L [^{14}C]-butyrate for 5 minutes (uptake) or 90 minutes (oxidation) at 37°C. For uptake measurements, cells were placed in 100% ethanol for 30 minutes, and the radioactivity in the supernatants was counted by liquid scintillation. For measurement of butyrate oxidation,[18] the reaction was stopped by adding perchloric acid to the medium (final concentration 4%), and $^{14}CO_2$ was recovered hermetically in 3 mol/L NaOH for counting of radioactivity. Butyrate uptake and oxidation results were normalized to cell protein content.

Statistical Analysis

The normality of data distribution was analyzed by the Smirnoff-Kolmogorov test. The significance of differences was determined using the Mann–Whitney *U* test for nonnormally distributed data (patients and rats) and 1-way ANOVA for normally distributed data (cell line experiments). Nonnormally distributed data (patients and rats) are presented as median ± interquartiles (IQ). Normally distributed data (cell line experiments) are presented as mean ± SEM. Simple regressions were used for statistical correlations.

Results

MCT1 Expression Is Decreased During Intestinal Inflammation

We assessed MCT1 messenger RNA (mRNA) and protein expression in segments of rat colon with DSS-induced colitis and control rats. Western blot analysis of protein lysates showed that MCT1 protein levels were significantly lower in the cecum and proximal and distal colon of rats with DSS-induced colitis in comparison with controls ($P < .01$) (Figure 1*A*, *i* and *ii*). Similarly, reverse-transcription (RT) PCR analysis showed a significant decrease in MCT1 mRNA expression in these DSS-inflamed colonic segments as compared with normal tissues (Figure 1*B*). Expression of MCT1 protein and mRNA was also determined in colonic biopsy specimens of patients with IBD. MCT1 immunofluorescence staining of colonic tissue sec-

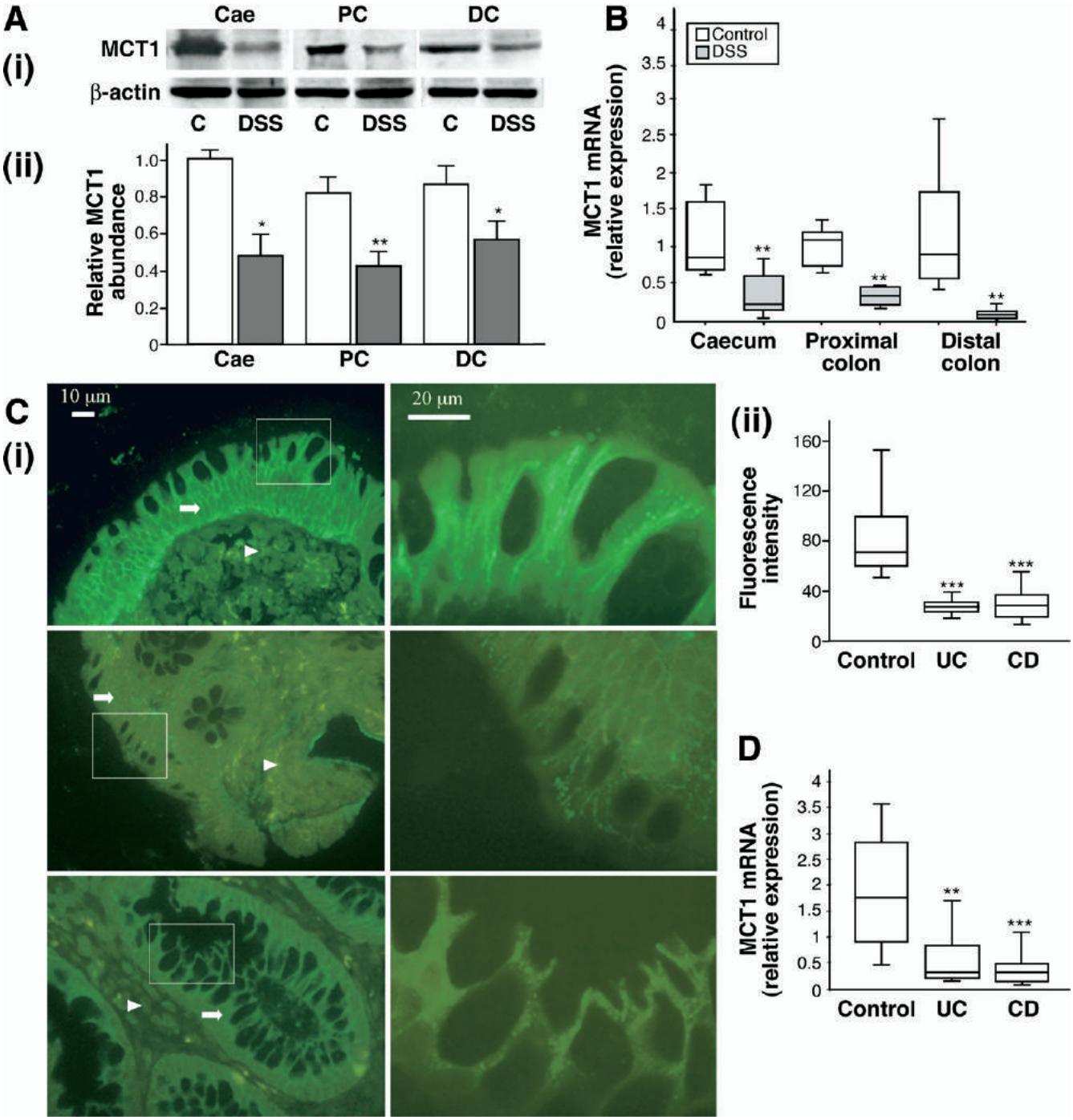

Figure 1. Effect of intestinal inflammation on MCT1 expression. (*A*) Western blot and (*B*) quantitative real-time RT-PCR analysis of MCT1 abundance in the cecum (*Cae*) and proximal (*PC*) and distal (*DC*) colon of control (*C*, n = 8) or DSS-treated (*DSS*, n = 8) rats. (*A*, *i*) Representative immunoblots of MCT1 and β-actin. (*A*, *ii*) Densitometric analysis of MCT1 protein expression (mean ± SEM, normalized to β-actin). (*B*) *Boxes* represent the median ± interquartiles of the relative MCT1 mRNA expression, and *error bars* indicate the tenth and ninetieth percentiles. **P* < .05; ***P* < .01 vs control. (*C*, *i*) Immunofluorescence staining of MCT1 protein in colonic sections from mucosa of healthy controls (n = 5) and patients with UC (n = 5) or CD (n = 5). *Arrows*, epithelium; *arrowheads*, lamina propria. *Right panels*, higher magnification of insets. (*C*, *ii*) Quantification of fluorescence intensity (median ± interquartiles) within the epithelium. (*D*) Quantitative real-time RT-PCR analysis of MCT1 mRNA expression in the colonic mucosa of healthy controls (n = 10) and patients with UC (n = 9) or CD (n = 14). *Boxes* represent the median ± interquartiles of the relative MCT1 mRNA expression, and *error bars* indicate the tenth and ninetieth percentiles. ***P* < .01; ****P* < .001 vs control.

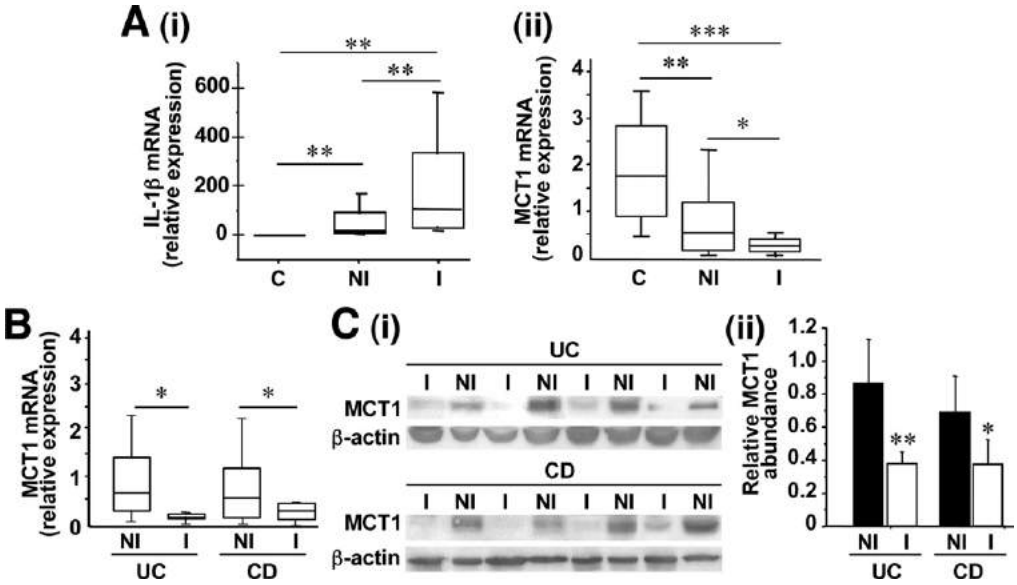

Figure 2. Effect of the degree of mucosal inflammation on MCT1 expression. (*A*) Quantitative real-time RT-PCR analysis of (*i*) IL-1β and (*ii*) MCT1 mRNA expression in inflamed (*I*) and noninflamed (*NI*) colonic mucosa of IBD patients (n = 23) and healthy controls (*C*, n = 10). (*B*) Quantitative real-time RT-PCR analysis of MCT1 mRNA expression in paired inflamed (*I*)/noninflamed (*NI*) colonic mucosa of patients with UC (n = 8) and CD (n = 10). *Boxes* represent the median ± interquartiles of the relative mRNA expression; *error bars* indicate the tenth and ninetieth percentiles. *$P <$ .05; **$P <$ .01; ***$P <$ .001 vs control. (C) Western blot analysis of MCT1 abundance in paired inflamed (*I*)/noninflamed (*NI*) colonic mucosa of patients with UC (n = 4) and CD (n = 4). (*C*, *i*) Representative immunoblots of MCT1 and β-actin. (*C*, *ii*) Densitometric analysis of MCT1 protein expression (mean ± SEM, normalized to β-actin). *$P <$ .05; **$P <$ .01 vs NI.

tions showed that MCT1 protein expression was dramatically reduced in inflamed mucosa of patients with UC and CD in comparison with healthy mucosa of controls (Figure 1*C*, *i*). In healthy subjects, MCT1 expression was almost exclusively confined to the epithelium. MCT1 staining was also found in cells of the lamina propria—probably immune cells—as recently reported.[19] Fluorescence quantification of MCT1 protein within the epithelium of patients with UC (median fluorescence intensity ± interquartiles, 27.0 ± 8.0) and CD (28.5 ± 17.5) showed lower MCT1 levels compared with normal controls (70.5 ± 36.5, $P <$ .001) (Figure 1*C*, *ii*). This finding suggests that MCT1 expression is reduced in the epithelial layer of the inflamed mucosa of IBD patients. Similarly, MCT1 mRNA levels were significantly lower in colonic biopsy specimens of patients with UC or CD than in normal colonic mucosa of controls (Figure 1*D*). No differences in these parameters were noted between UC and CD patients.

The Level of Decline in MCT1 Expression Is Dependent on the Inflammatory State of the Colonic Mucosa

We next sought to verify whether the down-regulation in MCT1 expression was proportional to the degree of mucosal inflammation. We first confirmed the endoscopic estimation of the inflammatory status of colonic biopsy specimens by RT-PCR analysis of IL-1β mRNA levels. As expected, IL-1β mRNA levels were significantly higher in inflamed biopsy specimens than in noninflamed or control biopsy specimens, being lowest in controls ($P <$ .01) (Figure 2*A*, *i*). In contrast, MCT1 mRNA levels were significantly decreased in inflamed biopsy tissues as compared with noninflamed ($P <$ .05) and control tissues ($P <$ .001) (Figure 2*A*, *ii*). MCT1 mRNA levels were also significantly decreased in noninflamed tissues compared with controls ($P <$ .01) (Figure 2*A*, *ii*). The reduction in MCT1 mRNA abundance, induced by inflammation, was similar irrespective of the regional origin of the biopsy specimen (data not shown). Analysis of paired samples from inflamed and noninflamed biopsy specimens from both UC (n = 8) and CD (n = 10) patients showed that MCT1 mRNA levels were significantly reduced in inflamed mucosa ($P <$ .05) (Figure 2*B*). Furthermore, Western blot analysis of protein extracts of the same biopsy specimens also showed decreased levels of MCT1 protein in inflamed colonic mucosa of both UC ($P <$ .01) and CD ($P <$ .05) patients in comparison with paired noninflamed mucosa (Figure 2*C*, *i* and *ii*). Collectively, these results provide evidence that, in IBD (both UC and CD), the decrease in MCT1 expression is closely related to the degree of mucosal inflammation.

Proinflammatory Cytokines Down-Regulate MCT1 Expression in Intestinal Epithelial Cells

Because we observed a markedly decreased expression of MCT1 in the inflamed colonic epithelium, we aimed to determine the potential of proinflammatory cytokines to down-regulate MCT1 expression in vitro. Therefore, we tested the effect of IFN-γ and TNF-α, alone or in combination, on the intestinal epithelial cell line

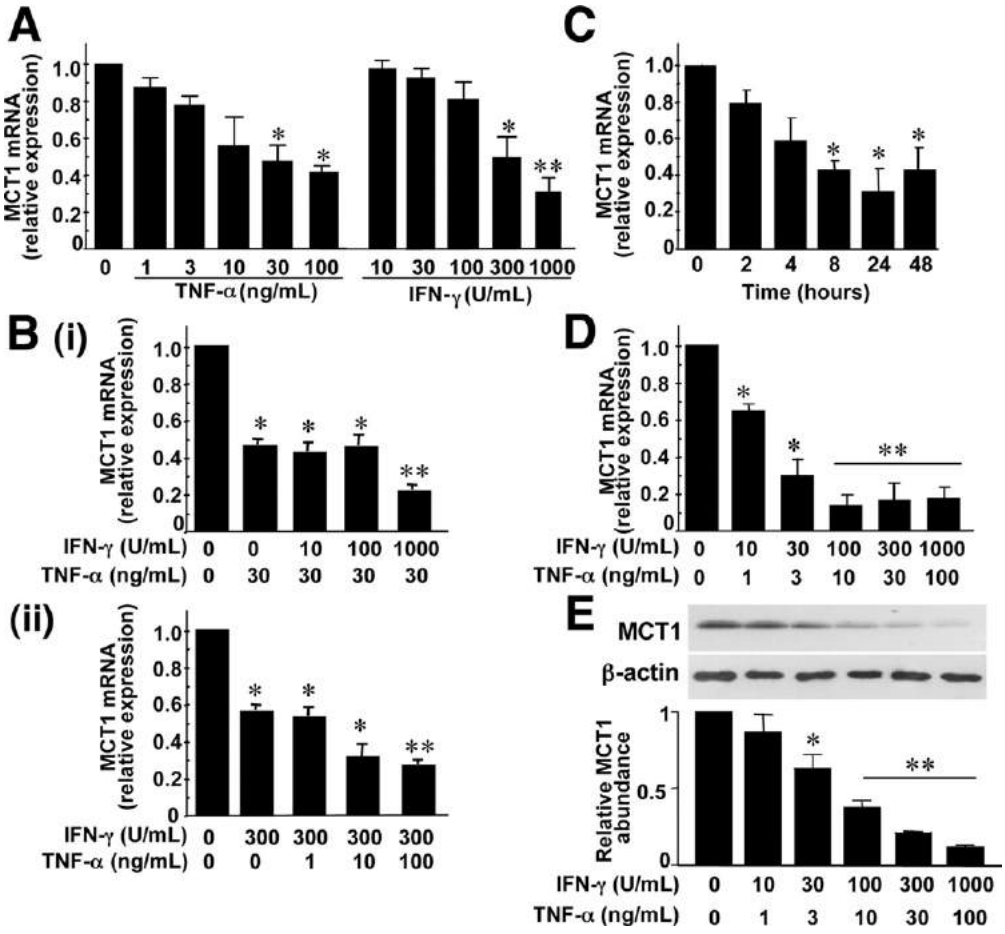

Figure 3. Effect of proinflammatory cytokines on MCT1 expression in the human intestinal epithelial cell line HT-29. (*A*) Quantitative real-time RT-PCR analysis of MCT1 mRNA abundance in HT-29 cells treated for 24 hours with increasing concentrations of IFN-γ or TNF-α. (*B*) Synergistic effect of IFN-γ and TNF-α on MCT1 mRNA expression in HT-29 cells treated for 24 hours at increasing concentrations. (*C*) Time-dependent changes in MCT1 mRNA expression in cells treated with 300 U/mL IFN-γ plus 30 ng/mL TNF-α. (*D* and *E*) Dose-dependent decrease in MCT1 mRNA (*D*) and protein (*E*) abundance in HT-29 cells treated for 24 hours with increasing concentrations of 10:1 ratio of IFN-γ and TNF-α. (*E*) *Top:* representative immunoblot; *bottom*: combined densitometric analysis. Histograms represent the mean ± SEM of 3 independent experiments. Results are expressed relative to controls, assigned a value of 1 (normalized to β-actin). *$P < .05$; **$P < .01$ vs control.

HT-29. Quantitative RT-PCR analysis of cells treated with increasing concentrations of IFN-γ (10–1000 U/mL) or TNF-α (1–100 ng/mL) showed a dose-dependent decrease of MCT1 mRNA expression (Figure 3*A*). The half maximal effect was obtained for 300 U/mL IFN-γ ($P < .05$) and 30 ng/mL TNF-α ($P < .05$) (Figure 3*A*). The combination of either IFN-γ or TNF-α at these doses with increasing concentrations of each synergistically down-regulated MCT1 mRNA expression (Figure 3*B*, *i* and *ii*). The down-regulation induced by the cytokine mixture (300 U/mL IFN-γ plus 30 ng/mL TNF-α) was time-dependent with a maximal effect at 24 hours ($P < .05$) (Figure 3*C*). This effect was also observed with the intestinal epithelial cell lines Caco-2 and SW-1116 (data not shown). Furthermore, RT-PCR and Western blot analysis showed that treatment of HT-29 cells for 24 hours with increasing concentrations of cytokines (at 10:1 ratio) induced a dose-dependent decrease in MCT1 mRNA (Figure 3*D*) and protein expression (Figure 3*E*). For the maximal concentration of the cytokine mixture (1000 U/mL IFN-γ plus 100 ng/mL TNF-α), the decreases in MCT1 mRNA and protein levels were 75% and 87%, respectively ($P < .01$) (Figure 3*D* and *E*). These data suggest that proinflammatory cytokines down-regulate MCT1 predominantly at the level of mRNA abundance.

Proinflammatory Cytokines Down-Regulate MCT1 Gene Transcription

We have shown previously that modulation of the steady-state level of MCT1 mRNA can result from the regulation of both its transcription and stability.[17] Accordingly, we first investigated the effect of proinflammatory cytokines on the stability of the MCT1 transcript in HT-29 cells stimulated in the presence of the transcriptional inhibitor actinomycin D. The decay in MCT1 mRNA level, after inhibition of RNA synthesis, was similar in cytokine-treated and control cells (respective relative half-lives of MCT1, 6.1 vs 7.2 hours) ($P = .95$), indicating no effect of cytokines on the stability of the MCT1 transcript (Figure 4*A*). We next analyzed the effect of cytokines specifically on MCT1 gene transcription. Nuclear run-on reactions were performed using nuclei isolated from untreated or cytokine-treated HT-29 cells. In comparison with untreated controls, cytokine treatment strongly reduced the abundance of MCT1 nascent transcripts ($P < .01$) (Figure 4*B*). For the maximal cytokine concentrations, the magnitude of decrease in MCT1 mRNA (75%), as estimated by real-time RT-PCR (Figure 3*B*), is accounted for by the specific decrease (67%) in MCT1 transcription as determined by run-on assay (Figure 4*B*). Together, these data suggest that the cytokine

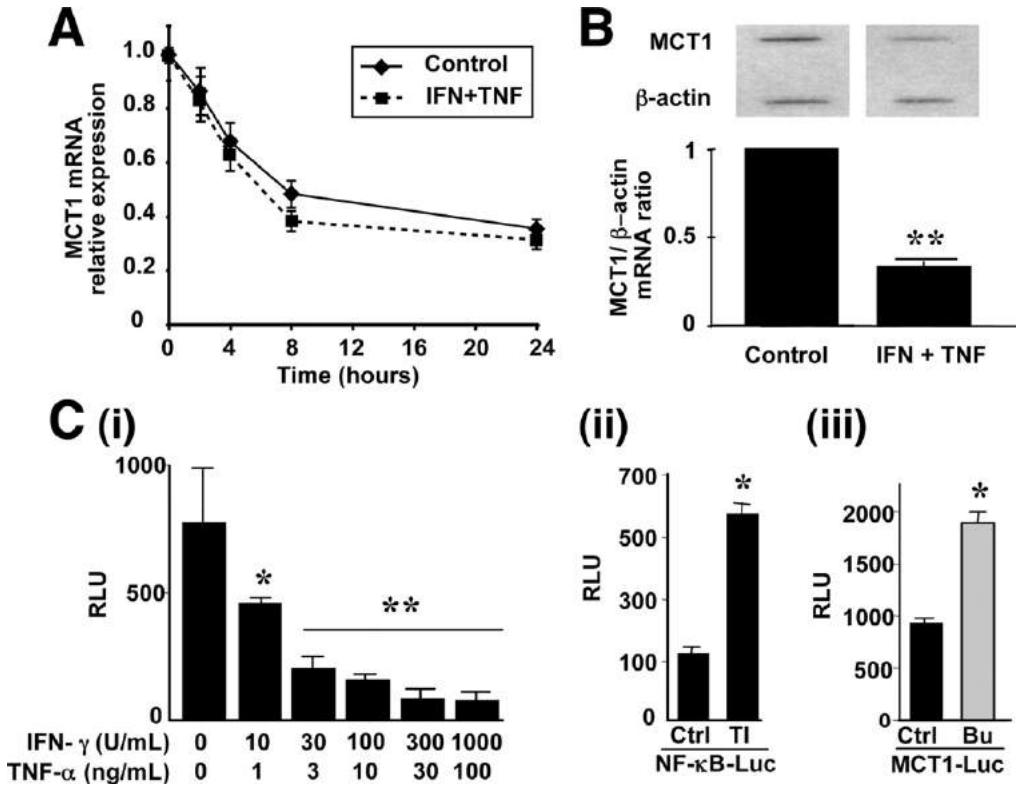

Figure 4. Effects of proinflammatory cytokines on MCT1 mRNA stability and transcription. (*A*) Real-time RT-PCR analysis of MCT1 mRNA stability. Control (*solid line*) and cytokine-treated (300 U/mL IFN-γ plus 30 ng/mL TNF-α) (*dotted line*) HT-29 cells were incubated in the presence of 1 μg/mL actinomycin D. Total RNA was extracted at indicated time points. Results are expressed relative to MCT1 mRNA abundance before actinomycin D treatment (time 0), assigned a value of 1 (normalized to β-actin). (*B*) Nuclear run-on analysis of MCT1 transcription. Control and cytokine treated (1000 U/mL IFN-γ plus 100 ng/mL TNF-α [*IFN + TNF*]) HT-29 cells were incubated for 24 hours. Nuclear run-on assays were performed on isolated nuclei. Nascent MCT1 mRNA transcription was normalized to β-actin. Histogram represents mean ± SEM of 3 independent experiments. Results are expressed relative to controls, assigned a value of 1. $^{**}P < .01$ vs control. (*C*) HT-29 cells were transfected with the pGL3-basic luciferase reporter plasmid containing the −1525/+213 upstream sequence of the MCT1 promoter or a specific NF-κB-reporter (NF-κB-Luc) plasmid. (*C*, *i*) Dose-dependent down-regulation of MCT1 promoter activity by proinflammatory cytokines. Transfected cells were treated for 24 hours with increasing doses of IFN-γ and TNF-α (as indicated). (*C*, *ii* and *iii*) Transfected cells were left untreated (*Ctrl*) or treated with (*ii*) TNF-α (30 ng/mL) plus IFN-γ (300 U/mL) (*TI*) or (*iii*) 5 mmol/L butyrate (*Bu*) for 24 hours. Luciferase activity values were normalized to EGFP fluorescence expressed by the cotransfected pIRES-EGFP vector. *Histograms* represent mean ± SEM of 6 independent experiments. *RLU*, relative light units. $^{*}P < .05$; $^{**}P < .01$ vs control.

mixture has a repressive effect on MCT1 transcription. To confirm this proposition, HT-29 cells were transfected with a luciferase reporter plasmid containing the −1525/+213 sequence of the human MCT1 promoter region[15] or an NF-κB-Luciferase reporter vector to be used as a positive control for the action of proinflammatory cytokines. Treatment of cells with increasing concentrations of IFN-γ and TNF-α (10:1 ratio) drastically reduced MCT1 promoter activity ($P < .01$) (Figure 4*C*, *i*). In contrast, treatment with IFN-γ (300 U/mL) plus TNF-α (30 ng/mL) stimulated NF-κB-reporter gene activity (Figure 4*C*, *ii*). Furthermore, cells were also stimulated by butyrate as a positive control for transcriptional stimulation of MCT1.[17] Contrary to the inhibitory effect observed with cytokines, treatment of cells with butyrate stimulated MCT1 promoter activity (Figure 4*C*, *iii*). Therefore, run-on and reporter gene assays clearly support the proposition that cytokines repress MCT1 gene transcription.

To eliminate further any potential involvement of cell death in cytokine-induced down-regulation of MCT1, we performed MTT viability cell assays in cells treated with increasing concentrations of IFN-γ and TNF-α for 24 hours. Whatever the dose of cytokines, no loss in cell viability was observed (see Supplementary Figure 1*A* online at www.gastrojournal.org). As controls, we showed that treatment of cells for 24 hours with 10 mmol/L butyrate, a well-known proapoptotic agent, induced approximately 38% loss in cell viability ($P < .05$) (see Supplementary Figure 1*B* online at www.gastrojournal.org). However, butyrate treatment also stimulated MCT1 expression ($P < .05$) (see Supplementary Figure 1*C* online at www.gastrojournal.org), consistent with previous observations.[17] Moreover, staining of HT29 cell nuclei with Hoechst showed that treatment of cells with 1000 U/mL IFN-γ plus 100 ng/mL TNF-α did not induce chromatin condensation, a typical sign of apoptosis. On the contrary, 10 mmol/L butyrate treatment of HT29 cells did

induce chromatin condensation (see Supplementary Figure 2 online at www.gastrojournal.org), in accord with the loss in cell viability. Collectively these data show that cytokine-induced MCT1 down-regulation is not a consequence of cytokine-induced cell death.

The Proximal Region of the MCT1 Promoter Is Potentially Involved in Cytokine-Induced Gene Repression

We have previously cloned and characterized ~1.5 kilobases of the human MCT1 gene promoter.[15] To investigate the potential role of transcription factors involved in the cytokine-induced inhibition of MCT1 promoter activity, we transfected HT-29 cells with luciferase reporter plasmids containing successive deletions of the MCT1 promoter. Treatment of cells with 300 U/mL IFN-γ and 30 ng/mL TNF-α resulted in the reduction of luciferase activity in all constructs used ($P < .01$) (Figure 5*A*). Thus, we propose that there is (are) potential binding sequence(s) within the −111/+213 promoter region that could mediate the repressive effect of cytokines.

Several NF-κB binding sites are present in the MCT1 promoter region that could be the target of proinflammatory cytokines. It has been shown that NF-κB can also act as a repressor of gene transcription.[20,21] To determine whether NF-κB is involved in the cytokine-induced down-regulation of MCT1 transcription, HT-29 cells were stimulated with 300 U/mL IFN-γ plus 30 ng/mL TNF-α in the presence of the specific NF-κB inhibitor CAPE.[22] RT-PCR analysis revealed that, although CAPE slightly, but not significantly, decreased basal MCT1 mRNA levels in control cells, it did not prevent the decrease in MCT1 mRNA transcription in cytokine-stimulated HT-29 cells ($P < .01$) (Figure 5*B*, *i*). As a positive control for NF-κB inhibition, we verified that CAPE effectively prevented cytokine-induced IL-1β mRNA expression (Figure 5*B*, *ii*). Thus, the repressive effect of proinflammatory cytokines on MCT1 gene transcription does not involve NF-κB.

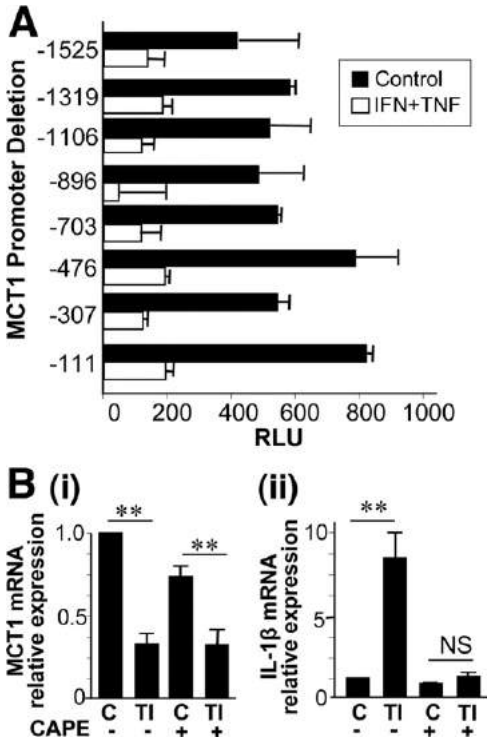

Figure 5. Mechanism of cytokine-induced down-regulation of MCT1 expression. (*A*) HT-29 cells were transfected with pGL3-basic luciferase reporter plasmids containing successive deletions (as indicated) of the MCT1 promoter. Control and cytokine-treated (30 ng/mL TNF-α plus 300 U/mL IFN-γ) cells were incubated for 24 hours before cell lysates were assayed for luciferase activity. Luciferase activity values were normalized to EGFP fluorescence expressed by the cotransfected pIRES-EGFP vector. *Histogram* represents mean ± SEM of 4 independent experiments. $P < .01$ vs control for all constructs. *RLU*, relative light units. (*B*) Inhibition of MCT1 mRNA expression by cytokines does not involve NF-κB. HT-29 cells were pretreated either with (+) or without (−) the NF-κB inhibitor CAPE (10 μmol/L). Control and cytokine-treated (30 ng/mL TNF-α plus 300 U/mL IFN-γ [*TI*]) cells were then incubated for 24 hours before quantitative real-time RT-PCR analysis of (*i*) MCT1 mRNA expression and (*ii*) IL-1β mRNA expression. Inhibition of cytokine-induced IL-1β mRNA expression by CAPE was used as a positive control for NF-κB inhibition. Histograms represent mean ± SEM of 3 independent experiments Results are expressed relative to control (−CAPE), assigned a value of 1. $^{**}P < .01$ vs control; NS, not statistically significant.

Impairment of Butyrate Uptake and Subsequent Oxidation in Intestinal Epithelial Cells Is a Consequence of MCT1 Down-Regulation

We next examined whether MCT1 down-regulation, induced by proinflammatory cytokines in HT-29 cells, is reflected functionally as a decrease in butyrate transport. Butyrate uptake and oxidation studies were performed in HT-29 cells by using [^{14}C]-labelled butyrate. Initial rate (5 minutes) of butyrate uptake in cells treated with increasing doses of IFN-γ and TNF-α was measured. As previously described,[11,12,17] kinetic studies, using a range of butyrate concentrations, showed that butyrate uptake exhibits typical Michaelis–Menten kinetics (data not shown). Eadie–Hofstee transformation of the data yielded a linear plot that was used for calculation of K_m and V_{max} (Table 1). Results show that, although the K_m of MCT1 remained essentially unchanged, V_{max} was significantly decreased in HT-29 cells treated with 100 U/mL IFN-γ plus 10 ng/mL TNF-α and 1000 U/mL IFN-γ plus 100 ng/mL TNF-α ($P < .01$). These data suggest that cytokine-induced reduction of butyrate uptake results from a reduction in transporter molecules rather than in transporter affinity for butyrate. We next analyzed the effect of proinflammatory cytokines on the steady-state rates of butyrate uptake and subsequent oxidation. Treatment of cells with increasing concentrations of IFN-γ plus TNF-α was associated with a dose-dependent decrease in MCT1 protein expression (Figure 6*A*, *i* and *ii*) and butyrate uptake ($P < .01$) (Figure 6*B*). This cytokine-

Table 1. Effect of Proinflammatory Cytokines on Kinetic Parameters of Butyrate Uptake in HT-29 cells

	Cell treatment			
	Control	T_1I_{10}	$T_{10}I_{100}$	$T_{100}I_{1000}$
K_m (*mmol/L*)	2.7 ± 0.5	2.5 ± 0.6	2.8 ± 0.6	3.0 ± 0.6
V_{max} (*nmol/min*)	36.6 ± 4.3	33.3 ± 4.2	22.4 ± 3.5[a]	14.9 ± 2.7[a]

NOTE. HT-29 cells were treated for 24 hours with TNF-α (T_1, 1 ng/mL; T_{10}, 10 ng/mL; T_{100}, 100 ng/mL) and IFN-γ (I_{10}, 10 U/mL; I_{100}, 100 U/mL; I_{1000}, 1000 U/mL). The initial rate (5 minutes) of butyrate uptake was calculated in the presence of a range of butyrate concentrations (0.1–10 mmol/L). Linear Eadie–Hofstee plots were used to determine K_m and V_{max}. Values are mean ± SEM of 5 independent experiments performed in triplicate.

[a]$P < .01$ vs control.

induced dose-dependent decrease of butyrate uptake was statistically correlated with the dose-dependent decrease of MCT1 protein levels ($R^2 = 0.956$; $P < .0007$) (Figure 6*C*) and mRNA levels ($R^2 = 0.932$; $P < .006$) (data not shown).

We further investigated whether cytokine-induced reduction of the intracellular levels of butyrate in HT-29 cells could lead to a decrease in butyrate oxidation rates. Immunofluorescence staining of MCT1 protein and measurements of butyrate uptake and oxidation were performed, in parallel, using HT-29 cells treated with or without 1000 U/mL IFN-γ plus 100 ng/mL TNF-α for 24 hours. Both x-y (Figure 7*A*, *i*) and x-z (Figure 7*A*, *ii*) images showed that treatment of cells with 1000 U/mL IFN-γ plus 100 ng/mL TNF-α strongly decreased plasma membrane expression of MCT1 (57% reduction in fluorescence intensity) compared with controls. The reduced expression of MCT1 was associated with a significant decrease in both butyrate uptake ($P < .05$) (Figure 7*B*) and subsequent oxidation ($P < .05$) (Figure 7*C*). Similar results were also obtained with polarized HT-29 cl.19A cells (data not shown). Our data suggests that (1) the decrease in butyrate oxidation observed in intestinal epithelial cells, under inflammatory conditions, is related to the decreased uptake of butyrate, not to a metabolic defect; and that (2) the down-regulation of MCT1 is directly responsible for the decreased rates of butyrate utilization by colonocytes during intestinal inflammation.

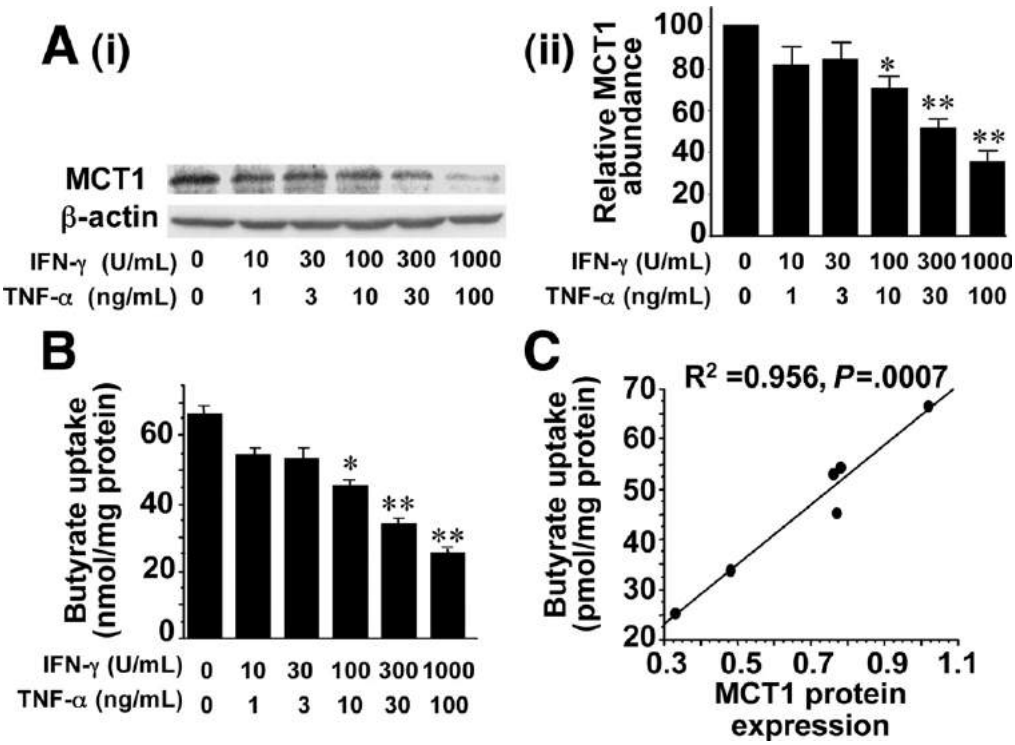

Figure 6. Down-regulation in MCT1 expression is correlated with reduced butyrate uptake. (*A*) Western blot analysis showing the dose-dependent decrease of MCT1 protein abundance in HT-29 cells treated with increasing concentrations of cytokines. (*A*, *i*) Representative immunoblots of MCT1 and β-actin. (*A*, *ii*) Densitometric analysis of MCT1 protein expression. Results are expressed relative to controls, assigned a value of 100 (normalized to β-actin). *$P < .05$; **$P < .01$ vs control. (*B*) [^{14}C]-butyrate uptake in HT-29 cells treated with increasing concentrations of cytokines. Results are expressed relative to protein concentration. *Histogram* represents the mean ± SEM of 3 independent experiments. *$P < .05$; **$P < .01$ vs control. (*C*) Linear correlation between MCT1 protein expression (*x-axis*) and butyrate uptake (*y-axis*) in HT-29 cells treated with increasing concentrations of cytokines. Regression coefficient (R^2) and its statistical level of significance are indicated.

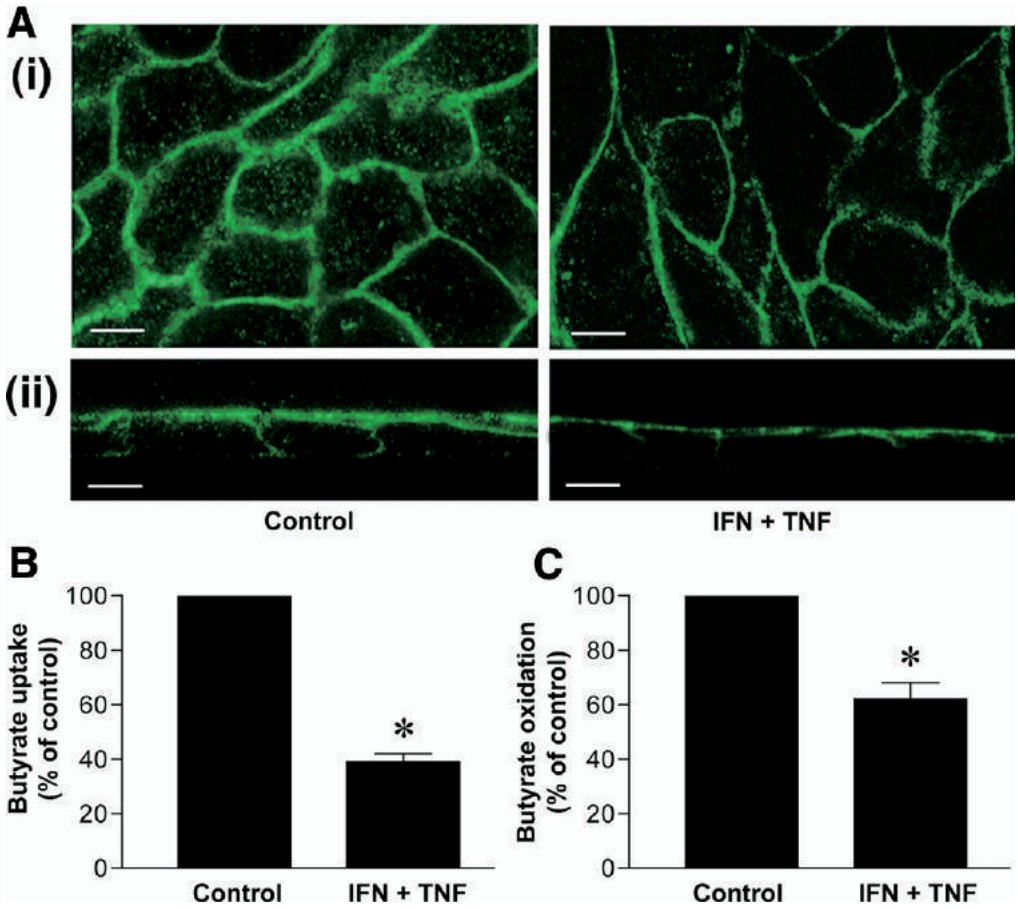

Figure 7. Decreased butyrate oxidation is the consequence of reduced butyrate uptake. (*A*) Immunofluorescence staining of MCT1 in control and cytokine-treated (1000 U/mL IFN-γ plus 100 ng/mL TNF-α [*IFN + TNF*]) HT-29 cells. Plasma membrane expression of MCT1 is markedly reduced by IFN + TNF treatment. (*A*, *i*) x-y (projection) and (*A*, *ii*) x-z confocal microscopy images of HT-29 cells (*scale bars*, 10 μm). (*B*) Butyrate uptake and (*C*) oxidation in cytokine-treated cells are expressed as percentage of control cells, assigned a value of 100%. *Histograms* represent the mean ± SEM of 3 independent experiments. *$P < .05$ vs control.

Discussion

Butyrate is essential to the health and integrity of the colonic mucosa, and its oxidation provides 70% of the total energy requirement of colonocytes. Previous studies in active IBD and in experimental DSS-colitis have shown that intestinal inflammation specifically affects butyrate metabolism because glucose and glutamine oxidation rates are not modified.[6,7,9,23] The data presented in this paper indicate that inflammation does not induce a general modification in colonocyte metabolism but affects regulation of butyrate transport. We hypothesize that a decline in butyrate oxidation may arise because of reduced intracellular butyrate availability. In this paper, we provide evidence that decreased expression of the butyrate transporter MCT1 in colonocytes by inflammation leads to a reduction in butyrate uptake and its subsequent oxidation.

We show that, in patients with IBD and in rats with DSS-induced colitis, colonic inflammation is associated with decreased MCT1 mRNA and protein levels. In vitro, we demonstrate that proinflammatory cytokines, which are abundantly produced during intestinal inflammation, down-regulate MCT1 expression in intestinal epithelial cell lines through the inhibition of its transcription. Furthermore, the reduction in MCT1 expression is reflected functionally as a decrease in butyrate uptake and subsequent oxidation. This is in accord with previous results showing that MCT1 down-regulation, using short interfering RNA (siRNA), led to a proportional reduction in butyrate uptake.[13] Together, our data support the proposition that the reduction in butyrate oxidation rates observed in the inflamed intestinal mucosa of IBD patients is related to decreased MCT1 expression and, hence, butyrate transport. Because the decrease in colonic MCT1 expression is a consequence of mucosal inflammation, our study suggests that butyrate oxidation deficiency in IBD is not a primary defect.[7]

Down-regulation of MCT1 is closely related to the intensity of colonic inflammation, as attested by the negative correlation observed between MCT1 and IL-1β mRNA levels, in inflamed, noninflamed, and normal mucosa. This is in agreement with previous reports showing that the endoscopically apparent noninflamed intestinal mucosa of IBD patients produces proinflammatory cytokines at levels intermediate of that produced by inflamed and healthy mucosa.[5,24] Interestingly, Roediger showed that butyrate oxidation rates in intestinal epithelial cells isolated from the colonic mucosa of patients with quiescent UC are at an intermediate level to the rates measured in healthy subjects and in patients with active UC.[6] Other studies, however, report that butyrate oxidation is not impaired in the colonic mucosa of patients with UC.[7,25,26] It is important to note that in those studies, colonocytes

were isolated from apparently normal mucosa of patients with moderate to severe disease or with quiescent disease. Indeed, no substantial deficiency of enzyme activity in the β-oxidation pathway was found in the mucosa of patients with UC in histologic remission.[27] In our study, the degree of inflammation in biopsy specimens was evaluated by RT-PCR analysis of IL-1β mRNA expression. Our findings, that expression of colonic MCT1 is closely related to the intensity of mucosal inflammation in IBD patients, may account for these conflicting results.

Several studies have reported that the expression of some membrane transporters, such as the amiloride-sensitive sodium channel ENaC, Na^+/K^+ ATPase (α1 and β1 subunits), and Na^+/H^+ antiporter (NHE3), are impaired in ulcerative colitis.[28,29] We have confirmed that these transporters are down-regulated concomitant with MCT1 in inflamed biopsy specimens of IBD patients (see Supplementary Figure 3 online at www.gastrojournal.org). Inhibition of MCT1 expression in HT-29 cells, using siRNA, had no effect on the expression profile of Na^+/K^+ ATPase (α1 and β1) or ENaC (see Supplementary Figure 4 online at www.gastrojournal.org). However, inhibition of MCT1 expression resulted in a concomitant increase in mRNA abundance and function of the glucose transporter isoform 1 (GLUT1) (see Supplementary Figures 4 and 5 online at www.gastrojournal.org), mimicking the in vivo correlation between MCT1 and GLUT1 expression observed in human colon during transition from normality to malignancy.[14] Therefore, it appears that inflammation affects the expression of a number of colonic membrane transporters.

High levels of proinflammatory cytokines are produced by the inflamed mucosa and are believed to play an important role in the pathogenesis of IBD. We show that treatment of intestinal epithelial cell lines with IFN-γ and TNF-α synergistically down-regulates MCT1 expression in a time- and dose-dependent manner. However, by blocking RNA transcription with actinomycin D, we show that proinflammatory cytokines do not increase the decay of the MCT1 transcript and, thus, do not affect MCT1 mRNA stability. However, using nuclear run-on assays, we provide evidence that proinflammatory cytokines down-regulate MCT1 expression at the transcriptional level. In addition, reporter gene experiments confirmed that cytokines inhibit MCT1 promoter activity.

The presence of NF-κB binding sites in the MCT1 promoter was potentially relevant to the transcriptional regulation of MCT1 during inflammation. NF-κB plays a key role in modulating the transcription of numerous genes during inflammatory responses and can also act as a repressor.[20,21] However, in our study, pharmacologic inhibition of NF-κB does not block the cytokine-mediated inhibition of MCT1 transcription. Moreover, transfection experiments with reporter plasmids containing successive deletions of the MCT1 promoter demonstrated that the repressive effect of proinflammatory cytokines involves potential *cis*-regulatory elements located within the −111/+213 promoter sequence. Further studies are underway to determine the specific transcription factor(s) involved in the cytokine induced down-regulation of MCT1 promoter activity.

In summary, our work suggests a correlation between MCT1 down-regulation and impaired butyrate utilization by colonocytes of inflamed intestine. A reduction in butyrate uptake affects the intracellular availability of butyrate. This has the potential to alter the ability of butyrate to exert its regulatory effects.[13] However, the relationship among inflammation, decreased MCT1 expression, and the development of colorectal cancer in IBD needs further investigation.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1053/j.gastro.2007.08.041.

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Received July 20, 2006. Accepted August 9, 2007.

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Part of this work was presented in abstract form at the DDW meeting, Chicago, May 17, 2005, and published in abstract form (Gastroenterology 2005;128[Suppl 2]:A497.

Supported by grants from the Ligue Nationale de Lutte contre le Cancer; the Biotechnology and Biological Sciences Research Council; and Tenovus, The Cancer Charity.

R.T. and P.d.C. contributed equally to this work.

We confirm that all of the authors have no conflicts of interest to disclose.