Amelioration of dextran sulfate colitis by butyrate: role of heat shock protein 70 and NF-κB

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Butyrate enemas have been demonstrated to ameliorate inflammation in ulcerative colitis. The mechanism of this protective effect of butyrate is not known, and this study examines the effect of butyrate on epithelial function, inducible heat shock protein 70 (HSP70) expression, and NF-κB activation in experimental colitis. Colitis was induced in rats by oral dextran sulfate sodium (DSS) and by butyrate or saline enemas. Mucosal barrier function was assessed by electrical resistance and [14C]mannitol permeability. HSP70 production was determined by [35S]methionine labeling, Western blot analysis, and immunohistochemistry. Activation of heat shock factors (HSFs) and NF-κB was evaluated by EMSA. Butyrate showed a significant protection against the decrease in cell viability, increase in mucosal permeability, and polymorphonuclear neutrophil infiltration seen in DSS colitis. Butyrate inhibited HSP70 expression in DSS colitis and also inhibited the activation of HSF and NF-κB. Thus butyrate enema was found to be cytoprotective in DSS colitis, an effect partly mediated by suppressing activation of HSP70 and NF-κB.

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ULCERATIVE COLITIS (UC) and Crohn’s disease (CD) are major forms of inflammatory bowel diseases in humans. Damage to colonic epithelial cells with impaired cell metabolism and barrier function is a hallmark of active UC (17). Due to the complex etiology of disease, therapy for UC and CD is difficult. However, short-chain fatty acids such as butyrate have been used with encouraging results in the treatment of UC. Butyrate, produced by bacterial fermentation of unabsorbed carbohydrate in the colon, regulates many epithelial cell functions in the colon. Butyrate enemas have been used in the treatment of UC as well as a variety of other diseases characterized by colonic inflammation, including diversion colitis and radiation proctitis (6, 19, 44). The therapeutic efficacy of butyrate could be explained on the basis of the fact that butyrate serves as the major source of energy for colonicocytes, increases mucosal proliferation, promotes cell differentiation, and improves barrier function (10). However, the cellular signaling events initiated by butyrate to carry out its protective effect have not been completely elucidated.

Heat shock response is a universal response to cell injury, which has been also documented in inflammatory bowel diseases and other human colonic diseases, suggesting a close link between heat shock protein (HSP70) expression and disease etiology (23, 37). The heat shock response is mediated by a family of chaperone proteins of which HSP70 is an important member. The heat shock cognate 70 is constitutively expressed in many cells, whereas inducible forms of HSP70 and HSP72 are synthesized in response to injury (5, 15). Butyrate has recently been reported to increase cellular levels of HSP25 but not HSP72 or HSC73 in rat IEC-18 cells in culture, and this was associated with partial protection against oxidant-induced injury (32). Although the induction of HSPs is generally considered to be protective (29, 46), it is now realized that there is a complex interaction between this response and activation of cytokines in the inflammatory process. In fact, recent articles by Cobb and colleagues (12) and Malhotra and Wong (25) discuss this heat shock paradox, where induction of heat shock in cells primed by inflammation can have damaging effects. These studies indicate that an integrating feature of the heat shock pathway and inflammation is the NF-κB pathway. The transcription factor NF-κB plays a central role in regulating the expression of a number of cytokines involved in immune and inflammatory responses (40). In both CD and UC, an increased mucosal synthesis of proinflammatory cytokines such as IL-1β, -16, -8, and -6, and TNF-α characterize the excessive local immune response (27). Activation of NF-κB has been demonstrated in mucosa of patients with UC (16), and butyrate has been shown to inhibit NF-κB activation in lamina propria macrophages of patients with UC (24).

Epithelial cell expression of HSP70 is enhanced in active UC (23). Heat shock response can induce activa-
tion of proinflammatory cytokines such as IL-6, and evidence from animal and human studies suggests that excessive production of IL-6 is involved in the pathogenesis of inflammatory bowel disease (20). The promoter regions of human IL-6 have also been shown to contain consensus-binding motifs for NF-kB (20). These observations led us to the hypothesis that the mechanism of protection by butyrate could be through modulation of HSP and NF-kB. This was tested in dextran sulfate sodium (DSS) colitis in rats, which is a recognized animal model resembling UC in many respects (11), including decreased colonocyte energy production (1) and impaired barrier function (42).

MATERIALS AND METHODS

Animal Protocols

Adult Wistar albino rats, weighing ~200 g, were administered DSS (~40,000 molecular mass, 19% sulfation, a kind gift from Professor S. N. S. Murthy, Allegheny University, Philadelphia, PA). DSS was mixed with rat chow in 4% concentration and fed to the experimental group, whereas control rats were pair-fed with chow not containing DSS. We (43) have earlier shown that epithelial cell viability and barrier function in rat colon were affected after 1 day of DSS administration, whereas ulceration developed after 5 days and fibrosis after 7 days of DSS administration. In initial studies, rats were fed DSS for 5 days and killed on the 6th day, and colonocytes were isolated for examination of the HSP response. In the studies reported here, animals were administered DSS only for 3 days because we were particularly interested in the early epithelial changes before establishment of ulceration and fibrosis (43). Early clinical changes in DSS-fed rats also included the presence of occult blood in the stool with no apparent weight loss or decreased appetite. Because butyrate is normally present in the rat colon from bacterial fermentation of fiber, and because we intended to test the effects of butyrate enemas in DSS colitis, the chow fed to rats was modified to minimize fermentable fiber in the colon and to reduce colonic butyrate concentrations. A purified fiber-free diet based on the American Institute of Nutrition (AIN)-93M diet (31) was fed for 3 days after which 4% DSS was added to the same diet, whereas controls received the AIN-93M diet without DSS. Animals received single, daily, rectal enemas of 3 ml Ringer solution containing either 140 mM NaCl, pH 7.4 (control), or 115 mM NaCl with 25 mM butyrate, pH 7.4 (test group) for 3 days, and were killed 1 day after the last enema. Colonocytes were isolated for viability assay and assessment of HSP70 production.

Colonocyte Isolation and Cell Viability

Animals were killed by ether overdose, and the colon was flushed with saline and removed. Colonocytes were isolated by using oxygenated Ca²⁺-free Krebs-Henseleit (K-H) solution containing 0.01 M ethylene diamine tetra acetic acid (1). Purity of colonocytes by this method was found to be >80%. Cell viability was measured by quantitating release of lactate dehydrogenase into the medium (18).

Mucosal Barrier Function

The barrier function of the distal colonic mucosa was assessed in short-circuited Ussing chambers by measuring electrical resistance and permeability to ¹⁴C-mannitol as previously described (42). ¹⁴C was measured in the withdrawn samples by liquid scintillation spectrometry by using a Rack Beta counter (LKB Wallac). Fluxes of mannitol were calculated by using standard formulas (42).

Assessment of Inflammatory Cell Infiltration

Paraffin-embedded sections (4-μm thick) of distal colon were stained with hematoxylin and eosin and examined under the ×40 high-power objective and the number of polymorphonuclear neutrophil (PMN) in 10 high-power fields was counted and expressed as PMN/10 high-power fields as described elsewhere (40). Nonoverlapping fields were used for counting.

Immunohistochemistry

Sections of colon were fixed in formalin and embedded in paraffin. Sections (5 μm) were cut from these blocks and floated onto poly-l-lysine-coated slides, dewaxed with xylene, and brought to water. The sections were covered with diluted (1:15,000) mouse anti-HSP70 monoclonal antibody (SPA-810; Stressgen, Victoria, BC, Canada) and incubated for 30 min at 23°C. This antibody is specific for the inducible form of HSP70. Initial studies were done by using another antibody to HSP70, H5147 (Sigma, St. Louis, MO), which reacts with both inducible and constitutive forms of HSP70, but were then repeated by using the more specific antibody for inducible HSP70. The sections were drained and covered with biotinylated rabbit anti-mouse antibody (DAKO) diluted 1:200. Endogenous peroxidase was blocked with 0.5% H₂O₂ in methanol. The sections were then covered with peroxidase-conjugated avidin (DAKO), developed with fresh diaminobenzidine solution containing H₂O₂, and counterstained with hematoxylin. The sections were dehydrated, cleared, mounted in Gum Dammar, and examined.

SDS-PAGE and Autoradiography

Isolated colonocytes were resuspended in K-H solution in the presence or absence of different concentrations of butyrate. To 250 μl of suspension, 60 μCi/ml of [³⁵S]methionine was added, and colonocytes were incubated at 37°C for 45 min. The cells were then centrifuged at 3,000 rpm for 3 min, and the cell pellet was resuspended in 150 μl K-H solution. Resuspended colonocytes were lysed by sonication of 2-μm amplitude for 2 min (MSE Soniprep 150). The protein concentration of the sonicated suspensions was assayed by Lowry’s method. Samples were initially boiled for 5 min in protein dissociation buffer (9% SDS, 16% β-mercaptoethanol, 15% glycerol, 1 M Tris, pH 6.7, containing 3 mg bromophenol). Equal amounts of protein were then loaded onto 10% SDS-PAGE gel. Ten percent separating gel contained 10% acrylamide, 0.1% SDS, 0.075% ammonium persulfate, 0.05% N,N,N',N'-tetramethylethylenediamine (TEMED), and 0.373 M Tris, pH 8.8. Stacking gel contained 5% acrylamide, 0.1% bis acrylamide, 0.1% SDS, 0.2% ammonium persulfate, 0.069% TEMED, and 0.138 M Tris, pH 6.8. Electrophoresis of the gels was carried out for 6 h at 120 V in buffer containing 0.2 M Tris, 0.192 M glycine and 0.1% SDS, pH 8.6. They were then stained overnight in Coomassie blue stain (methanol/acetic acid/water 50:10:40 vol/vol/vol containing 0.1% Coomassie brilliant blue R). Gels were destained, dried, and exposed to X-ray film at −80°C for 14 days.
days. The films were developed and scanned by using an HP scanner, and density of the protein bands was quantitated by using Scion Image for Windows (Scion 1998).

Western Blot Analysis

Western blot analysis was carried out as described by Towbin et al. (41). After electrophoresis, gels were immersed in transfer buffer (25 mm Tris, 192 mM glycine, 20% methanol) and blotted onto 0.45-μm nitrocellulose membranes (Millipore) by using a Bio-Rad power supply. The membranes were covered with mouse anti-HSP70 monoclonal antibody (SPA-810; Stressgen) diluted 1:5,000 with blocking solution and incubated for 2 h at 23°C. After being washed three times, the blot was incubated with goat anti-mouse antibody conjugated with alkaline phosphatase (Genei, Bangalore, India) diluted 1:1,000 with blocking solution for 2 h at 23°C. After further washing, alkaline phosphatase was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Genei) as substrate. All incubation procedures were performed under gentle agitation at 23°C. Experiments performed with other short-chain fatty acids acetate and propionate did not inhibit HSP or NF-κB synthesis; thus butyrate was used for further studies.

Gel Mobility Shift Assay

The gel or EMSA was carried out with two separate objectives: (1) to detect the binding of activated heat shock factors (HSF) in the cytoplasm of colonocytes from DSS colitis to heat shock element (HSE) DNA and (2) to detect the binding of translocated NF-κB in the nuclei of colonocytes from DSS colitis to the appropriate binding site of DNA.

**HSF-HSE binding.** Colonocytes were isolated as described earlier from control rats, colitic rats receiving saline enema, and colitic rats receiving butyrate enema. Cells were suspended in protein dilution buffer (20 mM Tris, pH 7.9, 150 mM KCl, 1 mM dithiothreitol, 10% glycerol, and 50 μg/ml bovine serum albumin) and quick frozen at −70°C. Cells were lysed with repeated freeze and thaw to get the protein extract. These extracts were then incubated at room temperature for 40 min with synthetic double-strand HSE oligonucleotide (containing bases −80 to −115 of the human promoter of the gene encoding HSP70), end-labeled with [γ-32P]ATP (LCP1; Brit) and a DNA end-labeling kit (model KT-6; Genei). These were then subjected to 6% non-denaturing gel electrophoresis, dried, and autoradiographed as described earlier. Experiments were done in parallel by using a mutated motif of NF-κB binding oligonucleotide, 5'-TGAGGCG-GACTTTCACAGGCG-3′ (GSN15; Genei), to establish the specificity of the interaction.

**Statistics**

A minimum of three animals was used for each experiment. All experiments were performed in triplicate. Colonocytes for the experiments were from individual animals. All numerical values were expressed as means ± SE. The two-tailed Mann-Whitney U-test was used to assess significance.
of differences between means of groups. P values <0.05 were considered statistically significant.

RESULTS

Colonocyte Viability in DSS Colitis and Effect of Butyrate

Colonocytes isolated from rats with DSS colitis that had received saline enemas showed significantly reduced cell viability measured as percent release of lactate dehydrogenase (56.1 ± 2.1%), compared with colonocytes from normal rats (89.6 ± 0.9%) (P < 0.01). Colonocytes from colitic rats that had received butyrate enemas showed significantly higher colonocyte viability (77.6 ± 0.9%) compared with those that had received saline enemas (P < 0.01) (Fig. 1).

Mucosal Barrier Function and Effect of Butyrate

Tissue electrical resistance, an indication of barrier function, was significantly lower in the colon of rats with DSS colitis receiving saline enemas (60.2 ± 0.7 \( \mu \)ohm·cm\(^{-2} \)·h\(^{-1} \)) compared with normal control rats (115.2 ± 2.0 \( \mu \)ohm·cm\(^{-2} \)·h\(^{-1} \)) (P < 0.01). Colons from rats administered butyrate enemas showed significantly higher tissue electrical resistance (102 ± 3.1 \( \mu \)ohm·cm\(^{-2} \)·h\(^{-1} \)) than those that received saline enemas (P < 0.01). Measured flux of [\(^{14}\)C]mannitol across the colonic mucosa, reflecting passive permeation, was significantly increased in DSS colitis (26.3 ± 1.5 \( \mu \)mol·cm\(^{-2} \)·h\(^{-1} \)) compared with normal control rats (8.3 ± 1.1 \( \mu \)mol·cm\(^{-2} \)·h\(^{-1} \)) (P < 0.01). Passive flux of mannitol was significantly reduced in colitic rats that had received butyrate enemas (10.3 ± 2.4 \( \mu \)mol·cm\(^{-2} \)·h\(^{-1} \)) compared with those that had received saline enemas (P < 0.005) (Fig. 1).

The lamina propria of the colon contained very few neutrophils in control animals (6.7 ± 1.1 per 10 hpf). Neutrophil infiltration in the lamina propria was significantly increased in rats with DSS colitis that had received saline enemas (36.4 ± 7.8 per 10 hpf) (P < 0.005), and this was markedly reduced in colitic rats that had received butyrate enemas (13.4 ± 2.5 per 10 hpf) (P < 0.05 vs. control and P < 0.01 vs. DSS saline) (Fig. 1).

HSP70 Synthesis in DSS Colitis and Effect of Butyrate

Colonic sections from normal rats did not stain for HSP70 by using a monoclonal antibody. On the other hand, colon from colitic rats showed intense staining for HSP70 in both surface and crypt epithelium. Mild focal staining was also observed in stromal cells (Fig. 2). The intensity of staining for HSP70 was less in rats treated with butyrate enemas compared with those that had received saline enemas (Fig. 2). Inhibition of

Fig. 2. Effect of butyrate enemas on the heat shock protein (HSP70) content of colonocytes in DSS colitis. A, B, and C show representative sections of immunohistochemical staining of the colon by using anti-HSP70 monoclonal antibody. A: normal control rat. B: rat with DSS colitis that had received saline enemas demonstrated intense staining for HSP70 especially in epithelial cells both on the surface and crypt of the colon, whereas a few stromal cells showed less intense staining. C: section of colon from rat with DSS colitis that had received butyrate enemas after induction of colitis. Immunohistochemistry revealed that epithelial cell staining for HSP70 was considerably less intense than in rats that had received saline enemas after induction of DSS colitis. Right: Western blot analysis of colonocyte extracts from rats by using anti-HSP70 monoclonal antibody. Equivalent amounts of colonocyte protein were loaded onto the gel. Lane A: normal control rats did not show inducible HSP70. Lane B: rats with DSS colitis that had received saline enemas showed significant amounts of inducible HSP70. Lane C: rats with DSS colitis that had received butyrate enemas showed significant reductions in the amount of HSP70.
HSP70 expression in DSS colitis by butyrate enemas was confirmed independently by Western blot analysis by using specific antibody to the inducible form of HSP70 (Fig. 2).

We also examined the effect of adding butyrate in vitro on colonocyte synthesis of neoproteins in DSS colitis. Because DSS colitis colonocytes were expected to contain formed HSP70 at the time of isolation, immunohistochemistry and Western blot analysis would not be appropriate to examine the effect of adding butyrate after isolation on HSP70 synthesis. Hence, incubation with [35S]methionine followed by SDS-PAGE and autoradiography, which would detect only protein newly formed after colonocyte isolation, was used. Colonocytes isolated from normal control rats did not show significant incorporation of [35S]methionine into new proteins during the 45 min of incubation. On the other hand, colonocytes isolated from rats with DSS colitis showed significant [35S]methionine incorporation into new proteins, the most prominent band being at the 70-kDa position (Fig. 3). This band reacted with HSP70 antibody after immunoblotting. When colonocytes from colitic rats were incubated in vitro with butyrate, inhibition of neoprotein production was noted generally and of the 70-kDa band, specifically. Scanning densitometry of these protein bands revealed that butyrate inhibited HSP70 formation by 58–68% compared with colonocytes not incubated with butyrate ($P < 0.001$) (Fig. 3).

Gel Mobility Shift Assays

HSFs bind to the DNA HSE to induce HSP70 production. The EMSA to detect DNA-protein binding by using radiolabeled HSE DNA and colonocyte lysates from animals with DSS colitis was carried out to determine whether butyrate would inhibit this binding. Cell extracts of colonocytes from control animals did not show any protein bands that bound radiolabeled HSE (Fig. 4). Colonocytes isolated from rats with DSS colitis exhibited a large radioactive band, indicating activation and binding of HSF to radiolabeled HSE, thus producing an electrophoretic mobility shift (Fig. 4). This shift was not found in colitic rats that had received butyrate enemas in vivo. The addition of butyrate in vitro to colonocytes from colitic rats also showed inhibition of HSP70 synthesis (Fig. 3).
inhibited the mobility shift, indicating that butyrate directly inhibited formation of the HSF/HSE complex.

Further experiments were undertaken to exclude the possibility that the anti-inflammatory effect of butyrate in DSS colitis was due to blocking of NF-κB activation. Nuclear extracts from the colonocytes of control rats did not bind the labeled NF-κB binding oligonucleotide in the gel mobility shift assay (Fig. 5), whereas colonocyte nuclear extracts from colitic rats (receiving saline enemas) showed significant NF-κB DNA binding activity, indicating colonocyte NF-κB activation in DSS colitis. The specificity of binding was demonstrated by the fact that binding was inhibited when 200-fold molar excess of unlabeled NF-κB binding oligonucleotide was added, or when a mutant NF-κB binding probe with one nucleic acid substitution was used. Binding was markedly inhibited by the administration of butyrate enemas to colitic rats and was also inhibited by the in vitro addition of butyrate (Fig. 5). The HSP70 inhibitor quercetin also inhibited activation of NF-κB both alone and in combination with butyrate.

**DISCUSSION**

Butyrate is a molecule that has a variety of effects with particular reference to colonic epithelial physiology and disease. Butyrate, alone or in combination with other short-chain fatty acids, has been used in the therapy of a number of diseases characterized by colonic mucosal inflammation including UC (34, 37, 45). It has been assumed that the protective effect of butyrate in these diseases results predominantly from influencing energy availability in the colonocyte, because it is the primary source of energy in this cell type (33). However, the cellular signaling events mediating the protective effect of butyrate in inflammatory bowel disease have not been elucidated. The present study examined the mechanism of butyrate-mediated cytoprotection in an animal model of colitis, namely DSS colitis in rats. This model has been characterized earlier and shows a number of characteristics resembling UC (11). In agreement to other earlier studies in DSS colitis (2) and TNBS colitis (8), butyrate clearly had a cytoprotective effect on colonic epithelial cells in DSS colitis as evidenced by increased cell viability and reduced mucosal permeability. These effects on the epithelial barrier could, by themselves, underlie the reduction of mucosal inflammation by butyrate.

Inflammation is a major component in the etiology of UC, and HSPs can activate the immune system in various ways to perpetuate inflammation and tissue damage (38). HSP70 is an inducible HSP produced in response to a variety of different cellular stresses and increased colonic expression of HSP70 has been found in the inflammatory bowel diseases (23, 39). However, no study to date has reported the effect of butyrate on HSP70 synthesis and expression in DSS colitis. The present study demonstrates that colonocyte production of HSP70 was markedly increased in DSS colitis, with immunohistochemistry showing that inducible HSP70 was predominantly expressed diffusely in colonocytes. Although a link of HSP70 expression to regenerative activity has been suggested by a study in ischemic bowel disease that found HSP70 immunoreactivity to be highest in viable crypt and surface epithelial cells adjacent to foci of necrosis (22), no such pattern was discernible in DSS colitis in the present study. There are likely several reasons for increased colonocyte expression of HSP70 in DSS colitis. Colitis results in the release of specific cytokines, some of which, such as IL-6 and TNF-α in addition to oxygen free radicals released during inflammation, are thought to induce HSPs. Cellular ATP depletion may lead to HSP70 remaining complexed to proteins, and therefore not available for recycling, leading to increased HSF activation (19). In DSS colitis, colonocyte oxidation of butyrate is markedly suppressed (1, 23, 39), presumably leading to reduced cellular ATP levels, and this may be yet another mechanism explaining increased colonocyte expression of HSP70.

Interestingly, treatment with butyrate resulted in an inhibition of this increased HSP70 synthesis. The immunohistochemical data and immunoblots using monoclonal antibodies from two different sources confirm that we were detecting HSP70 and that butyrate prevented the increase in HSP synthesis induced in colitis. Although most studies suggest that the heat
shock response provides protection against epithelial and mucosal injury in the intestine and colon, the role of cellular HSP70 activation in colitis remains unclear. Induction of the heat shock response before any injury has a protective effect, whereas activation of the heat shock response subsequent to a proinflammatory stimulus has been shown to have cytotoxic effects (7, 9, 13, 14). In intestinal epithelial cell lines, heat shock before exposure to lipopolysaccharide increased cell survival, whereas heat shock after exposure to lipopolysaccharide reduced enterocyte viability leading to cellular dysfunction and increased apoptosis (48). Thus it is becoming evident that increased expression of HSP can have protective effects, whereas activation of the heat shock response leads to apoptosis in endothelial cells (28), possibly by suppressing the initiation of effects on histone acetylation, potentially providing another mechanism for suppression of HSP70 synthesis by butyrate.

A major link between the heat shock response and cellular inflammatory responses has been found to be the transcription factor NF-κB (25), which is implicated in the regulation of a variety of genes during immune and inflammatory responses (4). NF-κB activation has been noted in inflammatory bowel disease in humans (30, 35) and in DSS colitis in mice (26). The decrease in mucosal PMN infiltration in colitic rats receiving butyrate enemas coupled with its effect on HSP70 prompted us to examine the effect of butyrate on this key transcription factor. Butyrate inhibited the activation of NF-κB both when administered in vivo as enemas and when added in vitro to colonocytes isolated from colitic rats and when given as an enema in vivo to colitic rats. Whether butyrate has a dual role, reducing inflammation and improving epithelial cell integrity and barrier function in DSS colitis in rats and that these effects are associated with (and are likely to be mediated by) inhibition of activation of inducible HSP70 leading to reduced activation of NF-κB. Further studies to examine this association may prove to be useful in the development of therapies for the treatment of inflammatory bowel disease and other diseases characterized by colonic mucosal inflammation.

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