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HDAC inhibition prevents NF-κB activation by suppressing proteasome activity: Down-regulation of proteasome subunit expression stabilizes IκBα

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Abstract

The short chain fatty acid (SCFA) butyrate (BA) and other histone deacetylase (HDAC) inhibitors can rapidly induce cell cycle arrest and differentation of colon cancer cell lines. We found that butyrate and the specific HDAC inhibitor trichostatin A (TSA) can reprogram the NF-κB response in colon cancer cells. Specifically, TNF-α activation is suppressed in butyrate-differentiated cells, whereas IL-1β activation is largely unaffected. To gain insight into the relationship between butyrate-induced differentiation and NF-κB regulation, we determined the impact of butyrate on proteasome activity and subunit expression. Interestingly, butyrate and TSA reduced the cellular proteasome activity in colon cancer cell lines. The drop in proteasome activity results from the reduced expression of the catalytic β-type subunits of the proteasome at both the protein and mRNA level. The selective impact of HDAC inhibitors on TNF-α-induced NF-κB activation appears to relate to the fact that the TNF-α-induced activation of NF-κB is mediated by the proteasome, whereas NF-κB activation by IL-1β is largely proteasome-independent. These findings indicate that cellular differentation status and/or proliferative capacity can significantly impact proteasome activity and selectively alter NF-κB responses in colon cancer cells. This information may be useful for the further development and targeting of HDAC inhibitors as anti-neoplastic and anti-inflammatory agents.

Keywords: NF-κB; IκBα; TNF-α; IL-1β; Butyrate; TSA; HDAC; Proteasome; β5; β1; β2; Caco-2

1. Introduction

Butyrate (BA) is a short chain fatty acid (SCFA) produced by bacterial fermentation of dietary fiber within the gastrointestinal tract. It is actively absorbed by intestinal epithelial cells and is the major luminal source of energy for colonocytes [1,2]. In addition to being a fuel source, butyrate has a number of other profound biological effects [3–6]. For instance, butyrate can readily induce apoptosis of transformed cell lines [7,8]. The ability for butyrate to induce apoptosis of cancer cells may contribute to the cancer preventive effects ascribed to dietary fiber [9–11]. Short chain fatty acids, particularly butyrate, can also suppress intestinal inflammation. Clinical trials have shown that topical butyrate applications alleviate symptoms in patients with mild and moderate ulcerative colitis [12]. Butyrate is also effective at treating other inflammatory conditions of the distal gastrointestinal tract [6,13,14]. In association, deficiencies in luminal butyrate production have been linked to colonic inflammation [9]. Such results suggest that butyrate may play an important role in regulating intestinal inflammation, as well as suppressing cellular transformation.

Biochemically, butyrate is a histone deacetylase (HDAC) inhibitor [15]. HDAC inhibitors in general have been noted for their ability to induce cell cycle arrest, differentiation and apoptosis of a wide spectrum of transformed cells. It has been proposed that HDAC inhibitors serve to normalize HDAC activity in transformed cells,

Abbreviations: SCFA, short chain fatty acid; HDAC, histone deacetylase; NF-κB, nuclear factor-κB; IκB, inhibitor-κB; IKK, IκB kinase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; BA, butyrate; TSA, trichostatin A; CT-L, chymotrypsin-like; T-L, trypsyn-like; PGPH, post-glutamyl peptide hydrolyzing; E-MEM, minimal essential media with Earle's salts; EMSA, electrophoretic mobility shift assay; Suc-LLVY-AMC, *N*-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin

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which often express elevated levels of certain HDAC proteins [16–19]. These findings have prompted clinical trials to assess the cancer therapeutic activity of HDAC inhibitors (such as SAHA and LAQ824) [20–22]. Gene expression profiles of cells treated with HDAC inhibitors have revealed that roughly 2% of the expressed genes are altered following HDAC inhibition [23]. Interestingly, within this population of genes, roughly half increase in expression, while the other half decreases. These selective changes in gene expression likely result from the enhanced acetylation of gene-regulatory transcription factors (e.g., GATA, p53, Sp1 and Sp3), in addition to the enhanced acetylation of histone proteins [24–27].

Recently, HDAC inhibitors have been reported to modulate the activity of the transcription factor NF-κB in a number of different cell types including colon cancer cell lines and macrophages isolated from the lamia propria of the colon [3,28–31]. NF-κB is a central mediator of the immune and inflammatory response and has been implicated in promoting tumorogenesis by protecting cancer cells from apoptosis [32]. Upon activation, NF-κB rapidly enhances the expression of proinflammatory genes such as cytokines and cell adhesion molecules, as well as genes involved in promoting proliferation, angiogenesis and cell survival [33–35]. The ability of butyrate and other HDAC inhibitors to modulate NF-κB activity coincides with its proposed cancer suppressing and anti-inflammatory activities.

NF-κB is regulated through the binding of inhibitory molecules collectively referred to as the IκB proteins [36,37]. IκB family members include IκBα, IκBε, IκBγ, IκBζ, Bcl-3, p105, p100, splicing variants IκBβ1 and IκBβ2 [37–42]. Perhaps the most important and well-characterized inhibitor of the IκB family is IκBα. It is the most abundant inhibitor overall, and is responsible for the rapid activation of NF-κB [43–47]. During NF-κB activation, IκBα is phophorylated by the IκB kinase (IKK) complex and subsequently ubiquitinated by the multisubunit E3 ubiquitin-ligation enzyme, $SCF^{\beta TrCP}$ [48,49]. Ubiquitinated IκBα is then rapidly degraded by the proteasome, releasing NF-κB to influence target gene expression [50–52]. It has been reported that butyrate's ability to suppress NF-κB activity depends in part on its ability to suppress cellular proteasome activity [30,53].

Here, we provide evidence that HDAC inhibitors butyrate and trichostatin A (TSA) suppress proteasome activity by down-regulating the expression of select proteasome subunits. This ultimately prevents the ubiquitin-mediated, proteasome-dependent degradation of IκBα limiting NF-κB activation. Furthermore, we find that HDAC inhibitors selectively interfere with the proteasome-dependent activation of NF-κB, while having little effect on the proteasome-independent pathway. These findings further classify the cellular activities of a promising new class of anti-inflammatory/anti-neoplastic agents.

2. Materials and methods

2.1. Cell culture and treatments

All cell lines were purchased from American Type Culture Collection (Manassas, VA). Caco-2 cells were propagated in minimal essential media containing 2 mM L-glutamine and Earle's salts (E-MEM) supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, streptomycin (50 mg/ml) and penicillin (50 U/ml). HT-29 and SW480 cells were propagated in McCoy's 5A medium supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids and antibiotics. All medium components were purchased from Invitrogen Life Technologies (Carlsbad, CA). All cell types were treated with a number of agents in this study. The concentration and source of these agents are as follows: TNF-α, 50 ng/ml, (R&D Systems, Minneapolis, MN); IL-1β, 4 ng/ml, (Promega, Madison, WI); sodium butyrate, 4 mM, (Sigma–Aldrich, St. Louis, MO); TSA, 2 μM, (Calbiochem, San Diego, CA); MG-132, 60 μM, (Calbiochem). The TNF-α and IL-1β concentrations used were selected because they generated a reproducibly high level of NF-κB activity in the cell lines examined. These conditions were selected to ensure maximal sensitivity for the detection of cellular pathways that down-regulate the NF-κB response.

2.2. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared based on a previously reported protocol with minor modifications as described in Inan et al. [3]. For the DNA binding assays, double-strand NF-κB DNA oligonucleotide (Promega) were end-labeled with [γ-^{32}] adenosine triphosphate (3000 Ci/mmol at 10 mCi/ml; Amersham Pharmacia, NJ) using T4 polynucleotide kinase. Binding reactions were performed by mixing 7.5 μg of nuclear extract (in 7.5 μl) with 2.5 μg poly-dIdC and 1 μg bovine serum albumin to give a final volume off 14 μl. After 15 min incubation on ice, 40 fmol of labeled oligonucleotide (1 μl) was added to each reaction. Reactions were then transferred to room temperature for an additional 15 min. Reaction products were separated on a 4% polyacrylamide/Tris borate EDTA gel and analyzed by autoradiography. Antibody supershift and oligonucleotide competition experiments were performed to demonstrate the specificity of the EMSA results [3].

2.3. Immunoblotting

Cytosolic extracts were prepared as described in Inan et al. [3]. For immunoblotting studies, 25 μg of cytoplasmic protein (quantified by the Bio-Rad protein assay) was denatured under reducing conditions, separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels, and transferred to nitrocellulose by voltage gradient transfer.

The resulting blots were blocked with 5% non-fat dry milk. Specific proteins were detected with appropriate antibodies using enhanced chemiluminescence detection (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblotting antibodies used were: IκBα C-21, (Santa Cruz Biotechnology); subunit β1 PW8140, subunit β2 PW8145, subunit β3 PW8130, subunit β4 PW8890, subunit β5 PW8895, subunit β6 PW9000 and subunit β7 PW8135, (Affiniti Research Products Ltd., Mamhead, Exeter, UK); p21 C-19, (Santa Cruz Biotechnology); Ubiquitin P1A6, (Santa Cruz Biotechnology); Actin I-19 (Santa Cruz Biotechnology). The antibodies specific for ubiquitin and p21 were diluted 1:500 for immunoblotting. All other antibodies were employed at a 1:1000 dilution.

2.4. Proteasome activity assay

Proteasome activity in cellular extracts was quantified by using a fluorogenic proteasome-specific substrate. The assay is based on the detection of the fluorophore AMC (7-amino-4-methylcoumarin) after cleavage from the proteasome substrate Suc-LLVY-AMC (Calbiochem, San Diego, CA). Cytosolic extract (5 μg of total protein in 5 μl) was incubated in a 100 μl reaction containing 20 mM Tris–HCl (pH 7.8), 0.5 mM EDTA, 0.035% SDS and 70 μM Suc-LLVY-AMC for 15 min at room temperature. The change in fluorescence (substrate consumption) was measured over an interval of 45 min using a microtiter plate fluorometer (excitation, 360 nm; emission, 460 nm). Proteasome-independent activity was determined by performing the assay in the presence of proteasome inhibitor MG-132 (final concentration 60 μM) (Calbiochem). Proteasome activity values were derived by subtracting the fluorescence obtained in the presence of this inhibitor from the values obtained in its absence. The values shown represent the ratio of proteasome activity in each sample to that of the basal activity in the untreated extracts. Assays were performed in quadruplicate and statistical significance was determined by a paired Student's *t*-test.

Cellular proteasome activity was also estimated by quantifying the accumulation of polyubiquinated proteins by immunoblotting (Fig. 9A). This method allowed us to simultaneously estimate cellular proteasome activity and NF-κB binding. For this analysis, the MG-132-induced accumulation of ubiquitin-protein conjugates >85 kDa were quantified by scanning densitometry. The signal in the absence of MG-132 was set at 100% proteasome activity. Sixty micromolar MG-132 was presumed to cause complete inhibition since this concentration is >10,000-fold over the K_i (4 nM) and completely inhibited NF-κB activation by TNF-α [54].

2.5. Quantitative real-time PCR analysis

Quantitative real-time PCR was performed using the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) in conjunction with the iQ SYBR Green Supermix (Bio-Rad Laboratories). This supermix contains the DNA-binding dye SYBR Green that intercalates into the minor grooves of double-stranded DNA molecules allowing for detection. Total RNA was prepared by using Trizol LS reagent (Invitrogen). Five micrograms of total RNA from each sample was reverse transcribed into cDNA by Superscript II reverse transcriptase (Invitrogen). After reverse transcription, the cDNA was treated with 1 U RNase H at 37 °C for 20 min. Control reactions were performed using all components except reverse transcriptase in order to confirm the absence of genomic DNA contamination. One microliter of each RT reaction was mixed with the SYBR Green master mix and gene-specific primer sets (Table 1). PCR amplification was run at 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s after 15 min of initial denaturation at 95 °C for 45 cycles. Melting curves were performed to check the specificity of PCR products. Dilutions of the positive control (RNA from untreated samples) were prepared with the proteasome subunit β5 primer set and used for generating standard curves. The C_T (threshold cycle) value for each sample was determined by using the iCycler software. The values shown represent the ratio in gene expression as determined by dividing the C_T value of each sample by the C_T value corresponding to the expression for each product

Table 1
Gene-specific primer sets for real-time PCR analysis

Gene	5′ Up primer	3′ Down primer	Product size (bp)
Proteasome subunit β1	5′-cagaacaaccactgggtcct-3′	5′-cccggtatcggtaacacatc-3′	221
Proteasome subunit β2	5′-gcaactgaagggatggttgt-3′	5′-aaactagggctgcaccaatg-3′	241
Proteasome subunit β3	5′-atggtgactgatgactttgtgg-3′	5′-ctcgatgatgtggacaatgact-3′	183
Proteasome subunit β5	5′-taaggaacgcatctctgtagca-3′	5′-tccacttccaggtcataggaat-3′	240
Proteasome subunit β7	5′-gctcgaagatgaaccctttg-3′	5′-gaacgggcatctcggtagta-3′	250
Proteasome subunit β5i	5′-gggtcctggactctactacgtg-3′	5′-tacatattgacaacgcctccag-3′	210
Proteasome subunit α2	5′-ttcgtccatttggagtttcttt-3′	5′-gctgtatgaatggcatcttcaa-3′	193
Proteasome subunit α4	5′-agtgcggaagatctgtgctt-3′	5′-tgcttcagactggcgatgta-3′	174
Proteasome subunit α6	5′-gcgtcaggagtgtttggatt-3′	5′-gacatggctctgcagtcaaa-3′	216
18S rRNA	5′-aagcatttgccaagaatgtttt-3′	5′-ttaagtttcagctttgcaacca-3′	199
$p21^{WAF1}$	5′-ggcttcatgccaggctacttc-3′	5′-ccctaggctgtgctcacttc-3′	268

The T_M for each primer deviated from 60 °C by no more than 1 °C.

at basal levels in control samples. The ratios derived from the 18S rRNA transcripts were used to normalize all expression data. The 18S rRNA product was selected as an internal control as this gene transcript is generated from a distinctly different polymerase and less likely to vary under conditions that alter mRNA expression. All the samples, as well as the dilution standards, were run in triplicate (at least) and statistical significance was determined by a paired Student's *t*-test.

2.6. Neutral red uptake assay for apoptosis

The neutral red uptake assay is a modification of a method designed to measure lysosomal uptake as an estimate for viable cell number [55]. Caco-2 cells were plated on 60 mm tissue culture plates, grown to 80–85% confluency, and treated with either 4 mM butyrate or 2 μM TSA. Following treatments, the media was aspirated, replaced with fresh media containing 40 μg/ml neutral red and reincubated for an additional 1 h. The neutral red working media was then aspirated and the cells were washed twice with PBS. Adherent cells were detached from the tissue culture plates with trypsin/EDTA (TE) buffer and resuspended in 1 ml of PBS in preparation for flow cytometric analysis. Analysis was performed on a FACS-Calibur flow cytometer using the software Cell Quest (Becton Dickinson, San Jose, CA, USA). Forward and side scatter gates were set to include all viable cells.

3. Results

3.1. TNF-α-induced degradation of IκBα is proteasome-dependent in Caco-2 cells

Recent studies have indicated that not all activators of NF-κB induce the degradation of IκBα via the ubiquitin-proteasome system [56–59]. In fact, it has been reported that the proteasome does not play a predominant role in the IL-1β-induced activation of NF-κB in colonic epithelial cells [60]. Therefore, we sought to determine the involvement of the proteasome in the induced degradation of endogenous IκBα by proinflammatory cytokines TNF-α and IL-1β in the Caco-2 colonic epithelial cell line. As indicated in the immunoblots, both TNF-α (Fig. 1A) and IL-1β (Fig. 1B) induced the rapid degradation and subsequent re-emergence of IκBα in Caco-2 cells (panels marked −MG-132). The re-emergence of IκBα is facilitated by the NF-κB-dependent transcription of the IκBα gene, as well as the post-translational stabilization of the IκBα protein [43–47,61]. The immunoblots also reveal that the TNF-α-induced degradation of IκBα was inhibited by the proteasome inhibitor MG-132 (Fig. 1A). In contrast, proteasome inhibition (+MG-132) delayed the IL-1β-induced degradation of IκBα, but did not prevent its complete degradation (Fig. 1B). These results indicate that the TNF-α-induced degradation of IκBα in Caco-2 cells is proteasome-dependent, whereas the IL-1β-induced degradation employs a proteasome-independent pathway.

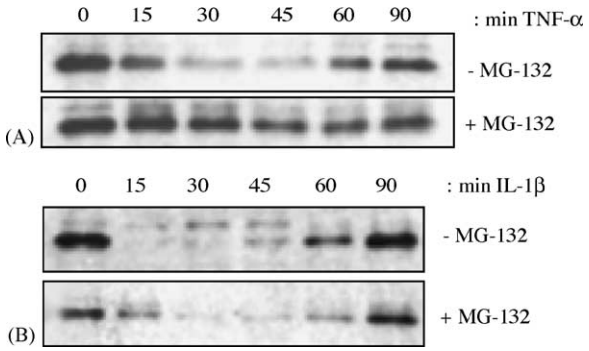

Fig. 1. Influence of proteasome inhibition on cytokine-induced IκBα degradation in Caco-2 cells. (A) Proteasome inhibition prevented the TNF-α-induced degradation of IκBα. Caco-2 cells were pretreated with (+MG-132) or without (−MG-132) proteasome inhibitor MG-132 for 30 min. Cells were then simulated with 50 ng/ml TNF-α for 0, 15, 30, 45, 60 and 90 min, as indicated. Cytosolic extracts were prepared and analyzed by immunoblotting using an antibody specific for IκBα. (B) Proteasome inhibition did not abrogate the degradation of IκBα-induced by IL-1β stimulation. Caco-2 cells were pretreated with (+MG-132) or without (−MG-132) MG-132 as mentioned in (A). Cells were then exposed to 4 ng/ml IL-1β for the indicated lengths of time in order to generate a time course capable of monitoring IκBα levels. Sample preparation and analysis were performed as in (A).

3.2. HDAC inhibition prevents the proteasome-dependent activation of NF-κB

Since both TNF-α and IL-1β induce the rapid degradation of IκBα in Caco-2 cells, we performed an EMSA to determine the affect of proteasome inhibition on NF-κB activation in Caco-2 cells. The data indicates that both TNF-α (Fig. 2A) and IL-1β (Fig. 2B) activated NF-κB DNA binding in Caco-2 cells. However, the proteasome inhibitor MG-132 was only capable of preventing the TNF-α-induced activation of NF-κB (Fig. 2A; +MG-132), while having a less pronounced effect on NF-κB activation induced by IL-1β (Fig. 2B; +MG-132). These results are in agreement with the data reported in Fig. 1, which demonstrated that IκBα degradation was predominantly proteasome-dependent in the presence of TNF-α (Fig. 1A), but largely proteasome-independent following IL-1β exposure (Fig. 1B).

Like MG-132, HDAC inhibitors have also been shown to suppress NF-κB activity in a signal-dependent manner [3,30]. We therefore determined the influence of HDAC inhibition on NF-κB activation in Caco-2 cells. As shown in Fig. 2A, pretreatment of Caco-2 cells with HDAC inhibitors butyrate or trichostatin A for 24 h blocked the TNF-α-induced activation of NF-κB in a manner similar to

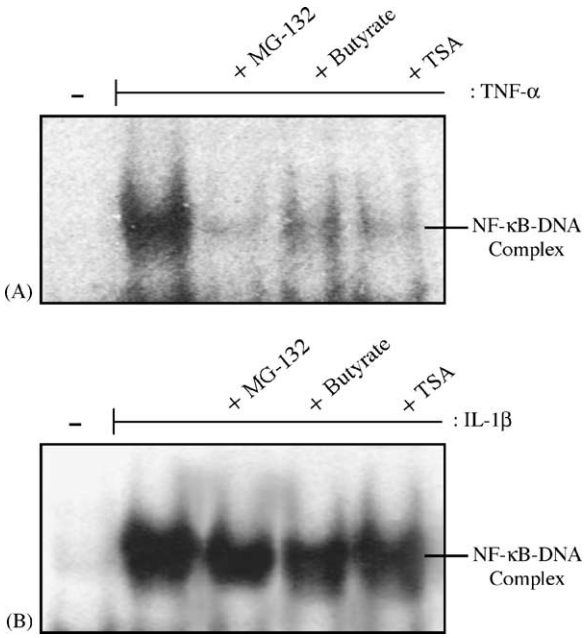

Fig. 2. Influence of proteasome and HDAC inhibitors on the cytokine-induced activation of NF-κB. (A) Both proteasome and HDAC inhibitors prevented the TNF-α-induced activation of NF-κB. Caco-2 cells were pretreated with proteasome inhibitor MG-132 (+MG-132) for 30 min, or with HDAC inhibitors butyrate (+Butyrate) or TSA (+TSA) for 24 h. After pretreatments, cells were exposed to 50 ng/ml TNF-α for 30 min. Nuclear extracts were then prepared and analyzed for DNA binding activity by EMSA. Control samples were also prepared from untreated (−) cells and cells exposed only to TNF-α. (B) The IL-1β-induced activation of NF-κB was not suppressed by either proteasome or HDAC inhibition. Caco-2 cells were stimulated with 4 ng/ml IL-1β for 30 min following MG-132 (+MG-132), butyrate (+Butyrate) or TSA (+TSA) pretreatments (as described in (A)). Controls consisted of untreated (−) cells and cells exposed only to IL-1β. Nuclear extracts were prepared and analyzed by EMSA as mentioned in (A).

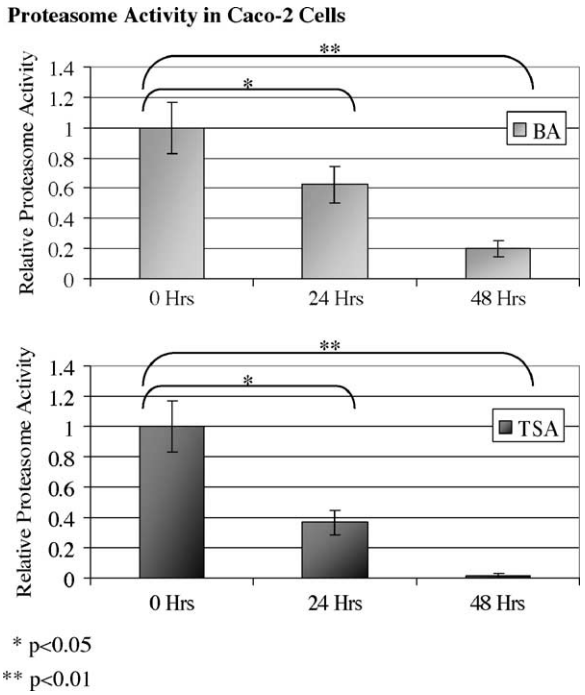

Fig. 3. HDAC inhibitors suppress proteasome activity in Caco-2 cells. Cell lysates were prepared from Caco-2 cells exposed to butyrate (BA) or TSA for 0, 24 and 48 h, as indicated. Proteasome activity was determined in each sample using a synthetic fluorogenic substrate (±standard error; $n = 4$). The reduction in proteasome activity was found to be significant at 24 ($^{*}p < 0.05$) and 48 h ($^{**}p < 0.01$) after initial butyrate and TSA treatments.

the proteasome inhibitor MG-132. In contrast, butyrate and TSA were unable to inhibit the activation of NF-κB in response to IL-1β (Fig. 2B). These results suggested that the HDAC inhibitors may selectively suppress proteasome-dependent NF-κB activation pathways.

3.3. HDAC inhibition suppresses proteasome activity

Since proteasome and HDAC inhibitors had a similar affect on NF-κB activation, cytosolic extracts prepared from control, butyrate and TSA treated Caco-2 cells were tested for proteasome activity using the synthetic substrate Suc-LLVY-AMC. As shown in Fig. 3, proteasome activity in Caco-2 cells decreased following HDAC inhibition. Within 24 h of exposure, proteasome activity dropped by ∼50% in both the butyrate and TSA treated samples. Proteasome activity continued to decrease and was reduced to ∼20% after 48 h of exposure. This data indicates that HDAC inhibitors butyrate and TSA suppress proteasome activity in Caco-2 cells, which likely contributes to the suppression of NF-κB activation following TNF-α treatments (Fig. 2A).

3.4. Proteasome subunit β5 expression is down-regulated by butyrate

Previous reports of microarry data have indicated that HDAC inhibitors can modulate the expression of genes involved in the ubiquitin-proteasome pathway [23,62]. One gene down-regulated following HDAC inhibition is the catalytic proteasome subunit β5. The β5-subunit is important for the rapid turnover of ubiquitinated-proteins [63–65]. We therefore decided to determine if HDAC inhibition by butyrate could also suppress the expression of β5 in Caco-2 cells. The immunoblot in Fig. 4A shows that β5 protein levels decrease in Caco-2 cells following butyrate treatment at 24 and 48 h in comparison to actin control levels. Since the level of β5 expression can impact cellular proteasome activity, the drop in β5 protein levels likely contributes to the decline in proteasome activity [66]. Fig. 4B confirms that β5 mRNA expression also decreases following butyrate treatment, as determined by real-time PCR.

Two other colonic epithelial cell lines were also examined to determine the influence of butyrate on β5-subunit expression. Cytosolic extracts were prepared from control and butyrate treated samples from each of the designated cell lines and analyzed by immunoblotting for the β5 protein. Fig. 4C indicates that β5 levels also decreased following a 24 h exposure to butyrate (BA) in the HT-29

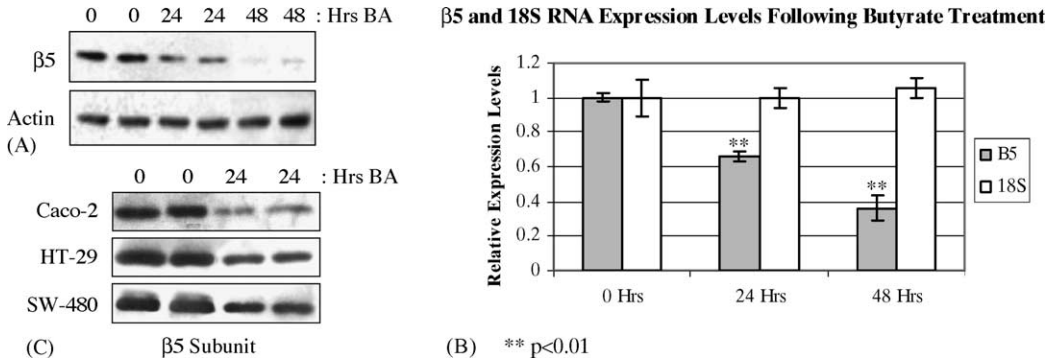

Fig. 4. Butyrate down-regulates β5 proteasome subunit expression. (A) Protein levels of the β5-subunit and actin following butyrate exposure. Caco-2 cells were treated with butyrate (BA) for 0, 24 and 48 h, as indicated. Cytosolic extracts were prepared and equivalent protein levels were analyzed using immunoblotting techniques. The levels of β5 protein were determined by using an antibody specific for the β5-subunit. Actin levels served as a loading control and were determined by immunoblotting for the actin protein. The results from duplicate cultures are shown. (B) β5 and 18S RNA expression levels following butyrate treatment. Caco-2 cells were treated with butyrate for 0, 24 and 48 h, as indicated. Total RNA extracts were prepared and 5 μg of RNA from each sample were reversed transcribed into cDNA. Quantitative real-time PCR was used in conjunction with gene-specific primers to determine relative β5 and 18S RNA expression levels (±standard error; $n = 6$). The reduction in β5 expression by butyrate at 24 and 48 h was found to be significant ($^{**}p < 0.01$) when compared to basal transcription levels in untreated samples (0 h). (C) Levels of β5 protein in Caco-2, HT-29 and SW-480 cells following butyrate exposure. Cytosolic extracts were prepared following 24 h treatment with butyrate (BA) in each cell line. Untreated samples (0 h) consisted of cytosolic extracts prepared from naïve cells. Equivalent protein levels were determined and analyzed by immunoblotting for the β5 proteasome subunit as described in Fig. 5A. The results from duplicate cultures are shown.

and SW-480 colon cancer cell lines. Although the degree of repression appears to vary between the cell lines, the general trend is the same.

3.5. HDAC inhibition decreases the expression of multiple proteasome subunits

To further explore the possible effects HDAC inhibition had on proteasome subunit expression in Caco-2 cells, we performed immunoblots to analyze the protein levels of each of the constitutively expressed β-type proteasome subunits following butyrate and TSA treatment. As Fig. 5 indicates, both butyrate and TSA caused a decrease in the protein levels of the three catalytic proteasome subunits: β5 (X), β1 (Y) and β2 (Z), (corresponding to the CT-L, PGPH and T-L activities of the proteasome, respectively). In contrast, little-to-no decrease in the protein levels of the other β-type subunits was observed.

We also sought to determine whether the down-regulation of the catalytic β-subunits occurred at the mRNA expression level. Real-time PCR was used to determine the expression levels of the indicated proteasome subunits following HDAC inhibition by butyrate and TSA for 24 h in Caco-2 cells. Both butyrate and TSA caused a significant decrease in the expression of the catalytic subunits β5, β1 and β2 (Fig. 6). This data indicates that HDAC inhibition decreases the expression of the catalytic proteasome subunits at the level of transcription. In agree-

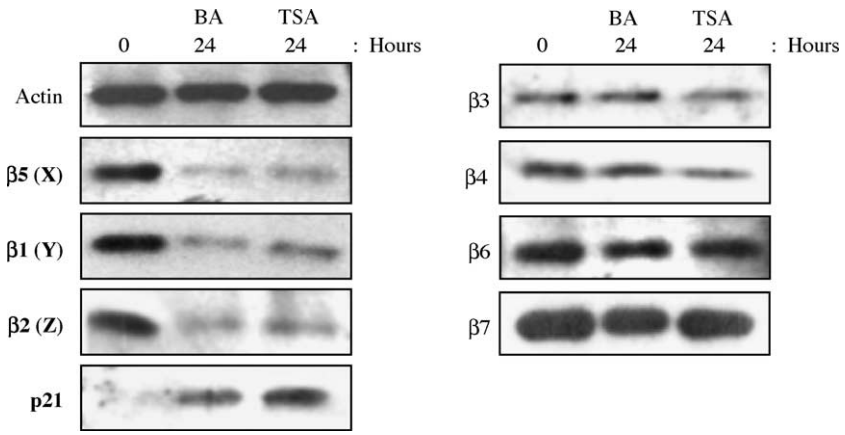

Fig. 5. Expression of the β-type proteasome subunits following HDAC inhibition; butyrate and TSA decrease levels of the proteasome subunits β5, β1 and β2 (referred to as the X, Y and Z catalytic components, respectively). Caco-2 cells were treated with butyrate (BA) or TSA for 24 h, as indicated. Control samples (0 h) consisted of untreated cells. Following cytosolic extraction procedures, immunoblot analysis was performed using antibodies specific for each of the constitutively expressed β-type proteasome subunits. Actin served as a loading control and was determined by immunoblotting for the actin protein. Protein levels of $p21^{WAF1}$ (p21) were also determined by immunoblot analysis to serve as an inducible control.

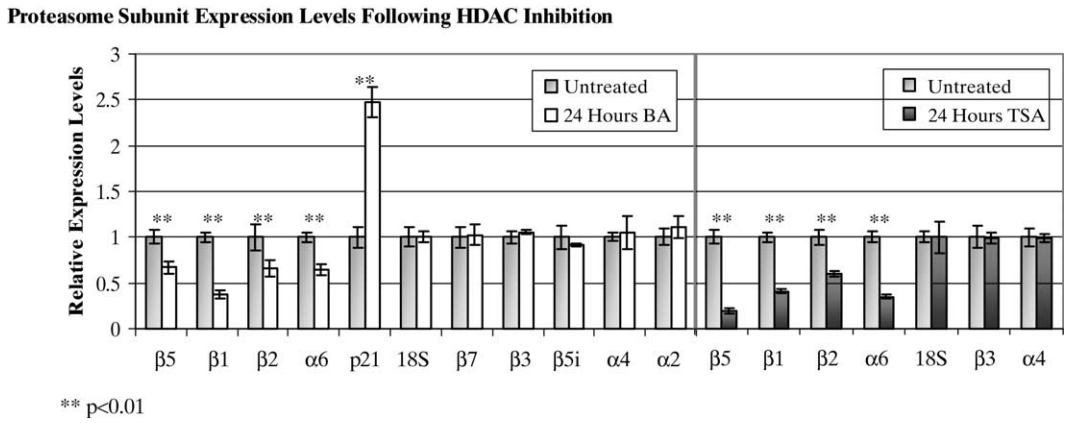

Fig. 6. Expression levels of various proteasome subunits following HDAC inhibition. Caco-2 cells were treated with butyrate (BA) or TSA for 24 h, as indicated. Untreated cells served as control samples to determine basal expression of each indicated transcript. Total RNA extracts were prepared and 5 μg of RNA from each sample were reversed transcribed into cDNA. Quantitative real-time PCR was used in conjunction with gene-specific primers to determine the relative expression levels of each indicated product (±standard error; $n \geq 3$). The modulations in transcription by butyrate and TSA were found to be significant ($^{**}p < 0.01$).

ment with the immunoblot data in Fig. 5, the mRNA levels of β3 and β7 were unaffected by the HDAC inhibitors.

Under certain conditions, the expression of alternate catalytic β-subunits can be induced to replace their constitutively expressed counterparts within the proteasome. These inducible subunits can ultimately modulate the activity of the proteasome [67,68]. The β5i (LMP7; PSMB8) protein is one such inducible subunit that can displace its β5 complement upon expression. To verify that the drop in β5 expression and proteasome activity by butyrate is not accompanied by a shift in proteasome subunit composition, we determined the expression of the inducible β5i-subunit following butyrate exposure. As the real-time PCR data indicates in Fig. 6, the expression of β5i did not change following a 24 h butyrate treatment. It should be noted that the basal expression levels of β5i were approximately 10 times less than that of β5 in Caco-2 cells. In association, β5i was not detectable

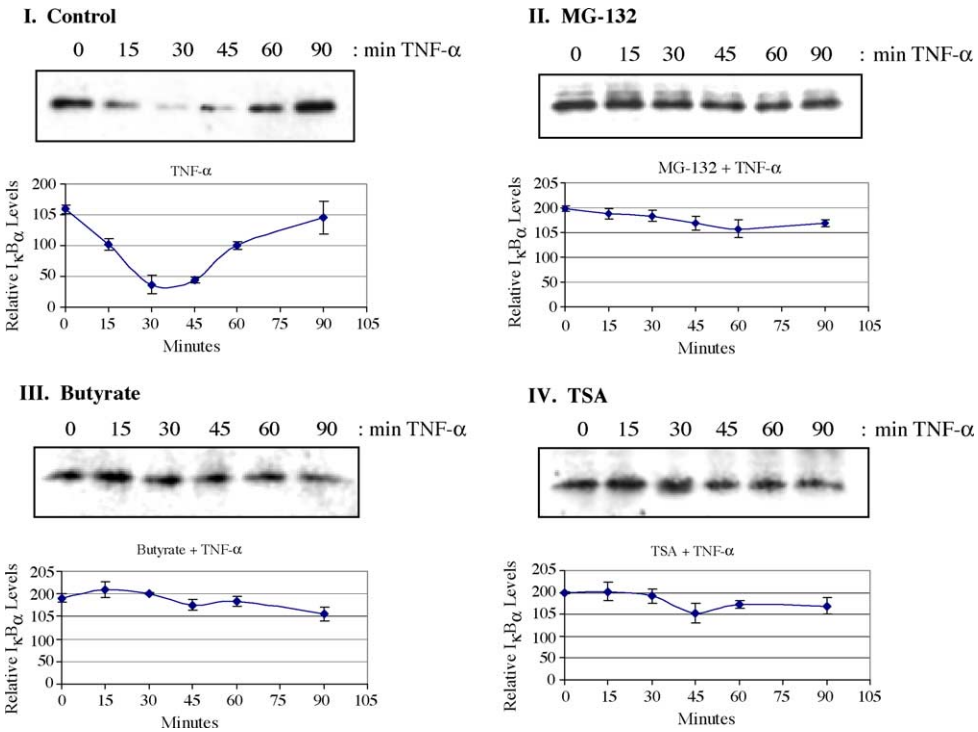

Fig. 7. HDAC inhibitors prevent the preoteasome-dependent degradation of IκBα. Caco-2 cells were exposed to TNF-α for the indicated lengths of time either alone (Panel I: control) or following pretreatments with MG-132 for 30 min (Panel II: MG-132), butyrate for 24 h (Panel III: butyrate) or TSA for 24 h (Panel IV: TSA). After treatments, cytosolic extracts were prepared and analyzed by immunoblotting using an antibody specific for IκBα. The graphical displays indicate the relative intensities of IκBα levels as determined by densitometry for each time point following TNF-α stimulation under the indicated treatment conditions (±standard error; $n = 3$).

by immunoblot under the described conditions both before and after butyrate treatment (data not shown). Such results imply that butyrate depletes the cell of the catalytic subunits, in particularly β5, to decrease cellular proteasome activity in absence of stimulating the expression of its β5i inducible counterpart.

The α-type subunits are constitutively expressed structural proteins composing the outer rings of the 20S proteasome. Although they are not catalytically active, they are responsible for limiting access of substrates to the inner catalytic β-rings [69]. We therefore, determined the expression of several α-type subunits to determine if butyrate could modulate their transcription, as well. As the real-time PCR data indicates in Fig. 6, expression of subunits α4 and α2 did not change following HDAC inhibition. However, transcription of the α6-subunit significantly decreased following butyrate and TSA treatments. The down-regulation of this subunit, in conjunction with the down-regulation of the catalytic β-subunits, may be limiting factors in proteasome biogenesis.

The cell cycle inhibitory protein $p21^{WAF1}$ was used as an inducible control in order to monitor gene activation as a result of HDAC inhibition. The promoter for $p21^{WAF1}$ is regulated by acetylation and has been routinely reported to be up-regulated following HDAC inhibition [70,71]. As the immunoblot in Fig. 5 indicates, butyrate and TSA readily induced the expression of $p21^{WAF1}$ protein (the panel labeled p21). Fig. 6 also indicates an increase in $p21^{WAF1}$ mRNA levels following butyrate exposure as determined by real-time PCR analysis.

3.6. HDAC inhibition prevents the proteasome-dependent degradation of IκBα

To determine if the HDAC inhibitors butyrate and TSA can modulate the proteasome-dependent degradation of IκBα in a manner similar to MG-132, we performed immunoblots to quantify IκBα protein levels during a TNF-α time course. As shown in Fig. 7, TNF-α rapidly induces the degradation of IκBα (Panel I: control). The proteasome inhibitor MG-132 blocks the degradation of IκBα in response to TNF-α (Fig. 7; Panel II: MG-132). HDAC inhibitors butyrate (Fig. 7; Panel III: butyrate) and TSA (Fig. 7; Panel IV: TSA) also prevent the degradation of IκBα similar to that of proteasome inhibitor MG-132. Taken together, this data indicates that both butyrate and

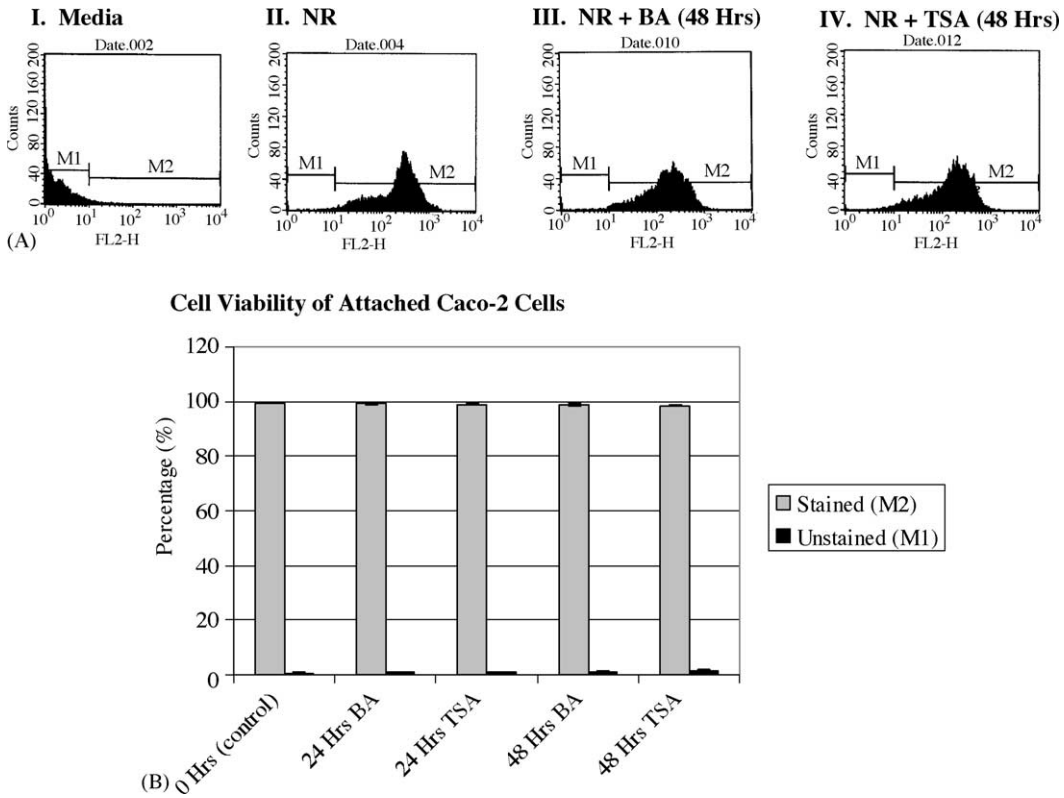

Fig. 8. Attached Caco-2 cells are equally viable following HDAC inhibition. Caco-2 cells were exposed to butyrate (BA) or TSA for 24 and 48 h. Control samples consisted of untreated cells. Before analysis, all floating cells were removed from the tissue culture plates and the remaining attached cells were stained with 40 μg/ml neutral red for 1 h. Attached cells were then processed for analysis by flow cytometry to measure the uptake of neutral red as a marker of cell viability. (A) Examples of histograms generated by flow cytometric analysis of attached Caco-2 cells stained with (NR) or without (Media) neutral red. Unstained cells were cropped to the M1 fraction, while stained cells resided in the M2 fraction. (B) Stained (M2) and unstained (M1) cells are represented as percentages of the total gated population of attached Caco-2 cells as determined for each indicated treatment condition (±standard error; $n = 3$). Control samples (0 h) consisted of untreated cells stained with neutral red.

TSA stabilize IκBα by preventing its proteasomal degradation in manner similar to MG-132. This action ultimately inhibits the proteasome-dependent activation of NF-κB (Fig. 2A).

3.7. Attached Caco-2 cells are equally viable following HDAC inhibitory treatments

Both butyrate and TSA increase the apoptotic rate of human colorectal carcinoma cell lines [72,73]. The samples used throughout these experiments were prepared from Caco-2 cells that remained attached to the tissue culture plates following butyrate and TSA treatments. The floating cells were washed off the tissue culture plates to eliminate the detached, dead cells from sample analysis. We performed the neutral red (NR) uptake assay on the remaining attached cells to determine the fraction of viable cells attached to the plate. Histograms generated from the flow cytometric analysis of cells are shown in Fig. 8A. Flow cytometric analysis of the attached cells after 48 h of butyrate or TSA treatments (Panels III and IV, respectively) indicated that majority of the cells resided in the viable M2 sector. Fig. 8B illustrates the percentages of stained (M2) and unstained (M1) Caco-2 cells under the different conditions. The percentage of stained (M2) attached cells remained nearly constant for each treatment (>95%). This data indicates that the drop in proteasome activity is not the result of lower cell viability.

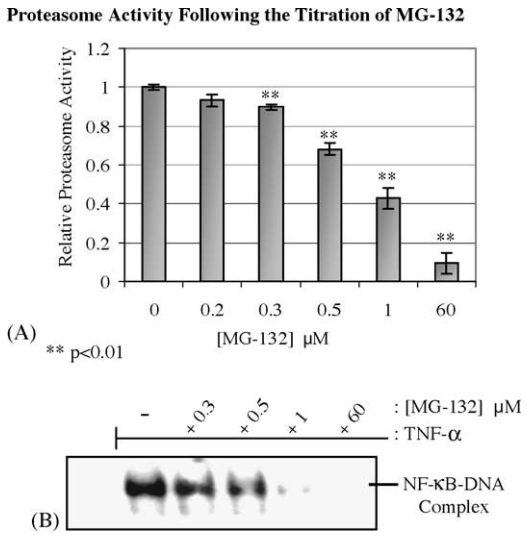

Fig. 9. Partial inhibition of the proteasome suppresses NF-κB binding activity. (A) Caco-2 cells were exposed to 0.2, 0.3, 0.5, 1 and 60 μM concentrations of MG-132 for 30 min. Control samples (0 μM) consisted of untreated cells. Relative proteasome activity was determined by optical densitometry measuring the accumulation in ubiquitin-conjugated proteins at the different concentrations of MG-132 from the resulting immunoblots (±standard error; $n = 3$). Reductions in proteasome activity were found to be significant ($^{**}p < 0.01$) at 0.3, 0.5, 1 and 60 μM concentrations of MG-132. (B) Caco-2 cells were pretreated with proteasome inhibitor MG-132 at the indicated concentrations for 30 min. Cells were then exposed to 50 ng/ml TNF-α for 30 min. Nuclear extracts were prepared and analyzed for DNA binding activity by EMSA. Control samples were also prepared from untreated (−) cells and cells exposed only to TNF-α.

3.8. Partial proteasome inhibition suppresses NF-κB activation

The HDAC inhibitors butyrate and TSA reduced cellular proteasome activity in Caco-2 cells by approximately 40–60% (Fig. 3), and suppressed the TNF-α-induced activation of NF-κB (Fig. 2A). To determine the sensitivity of NF-κB activation to a partial reduction in cellular proteasome activity, Caco-2 cells were incubated with concentrations of MG-132 that partially inhibited the proteasome. MG-132 is a specific, non-cleavable, competitive and reversible proteasome inhibitor with an in vitro K_i of 4 nM, although higher extracellular concentrations are required for proteasome inhibition in vivo (due to cellular partitioning) [54]. As shown in Fig. 9A, 0.5 and 1 μM MG-132 resulted in an estimated 30 and 60% reduction in cellular proteasome activity, respectively. An EMSA was then performed to determine whether partial proteasome inhibition with MG-132 affected NF-κB activation by TNF-α. As shown in Fig. 9B, NF-κB activation was found to be approximately proportional to the level of cellular proteasome activity. This finding further supports our model in which a reduction in catalytic proteasome subunit expression by HDAC inhibitors suppresses NF-κB activation by TNF-α.

4. Discussion

Numerous lines of evidence indicate that NF-κB promotes tumorigenesis [33,35]. The sustained activation of NF-κB suppresses apoptosis by increasing the expression of cellular survival signals. In fact, the increased activity of NF-κB has been found in many types of solid tumors and transformed cell lines, including those isolated from the colonic epithelium [74,75]. NF-κB also plays a pivitol role in the inflammatory response by inducing the expression of numerous inflammatory-related genes [33]. Recently, evidence has suggested that NF-κB is involved in the pathogenesis of chronic intestinal inflammation [76,77]. It has been proposed that through targeting NF-κB it may be possible to suppress inflammation and cancer development in the colon. In association, understanding how different anti-inflammatory and anti-neoplastic agents impact NF-κB could help target clinical application of these inhibitors and suggest effective combination treatments with other drugs.

In this report, we examine a mechanism by which butyrate influences NF-κB activation in the Caco-2 colonic epithelial cell line. Our analysis indicates that butyrate influences NF-κB activity by preventing the proteasome-dependent degradation of IκBα. This inhibition appears to arise from butyrate's ability to directly inhibit HDAC

activity, since similar results are obtained with the specific HDAC inhibitor, TSA. By down-regulated proteasome subunit expression, HDAC inhibitors can stabilize IκBα after TNF-α stimulation. Specifically, HDAC inhibition was found to down-regulate the expression of the three catalytic β-subunits: β5, β1 and β2. These subunits are solely responsible for all the endopepidase activities associated with the proteasome. The reduced levels in these catalytic subunits ultimately results in the global intracellular decline of proteasome activity.

It is unlikely that the reduction in cellular proteasome activity is the only mechanism by which butyrate and other HDAC inhibitors influence NF-κB activity. Recently, it has been shown that butyrate may also impair IKK activity [78]. Butyrate has also been reported to increase the levels of IκBβ and p100 in colon cancer cells [29,30]. However, our studies show that reduced expression of proteasome subunits has a major impact on NF-κB activation.

It is interesting to note that in some instances, HDAC inhibitors have been reported to prolong NF-κB activation [79]. This activity of HDAC inhibitors arises in part from its influence on the cytoplasmic-nuclear shuttling of IκBα. We have also noted that HDAC inhibitors can accentuate NF-κB activation in colon cancer cells when the proteasome-independent activator IL-1β is employed [3]. HDAC inhibitors therefore function to reprogram the NF-κB response, rather than simply inhibiting NF-κB activation. The complex actions of HDAC inhibitors on NF-κB activation need to be carefully considered prior to the clinical application of these agents.

Several studies have documented that HDAC inhibition results in the accumulation of ubiquitinated-protein conjugates [30,53,62,80]. We suspected from microarray analyses that these ubiquitinated-protein deposits were resulting from the reported decreases in the expression of the catalytic β5-subunit following HDAC inhibition [23,62]. The down-regulation of this subunit would suppress the CT-L activity of the proteasome, which would directly inhibit ubiquitinated-protein turnover [63,64]. Drops in β5 levels would also limit total proteasome content as this protein is necessary for proper proteasome assembly [66,81]. We report that both butyrate and TSA can specifically reduce the transcription of all three catalytic proteasome β-subunits with minimal influence on the expression of the non-catalytic β-subunits in Caco-2 cells. It has been suggested that a common mode of feedback regulation is shared amongst the catalytic subunits. In fact, it has been shown that β5 overexpression in human cell types selectively increases the expression of the other catalytic subunits [82,83]. Interestingly, a common mode of transcriptional regulation of the proteasomal subunits has already been identified in yeast [84,85]. Nearly all the yeast subunit homologs have been found to possess proteasome-associated control elements within their promoters. The transcription factor RPN4 has been identified as the component within yeast involved in binding these elements to modulate gene transcription [84]. It is thus possible to speculate that a common transcriptional mechanism is shared amongst the catalytic subunits in human cells that may be autoregulated by the proteasome and/or influenced by acetylation. It is interesting to note that NF-κB itself has been reported to regulate the expression of some α-type and 19S subunits [86]. However, we have not performed a detailed analysis of these components in colon cancer cells.

In association to the decline in the catalytic β-subunits, butyrate and TSA also reduced the transcription of the α6 proteasome subunit. The α6 protein is largest of the α-type subunits and is located in the outer rings of the 20S proteasome. Although it possesses no catalytic activities, it has been reported that this subunit can effectively modulating the activity of the proteasome. Antisense oligos directed towards the α6-subunit have resulted in the decreased degradation of proteasome-specific substrates [87]. Furthermore, the bacterial product LPS has been shown to directly bind α6 to increase the CT-L activity of the proteasome [88]. The observed decline in α6 expression following HDAC inhibition in Caco-2 cells may further contribute to the reduction in cellular proteasome activity. Another interesting fact is that the α6-subunit also contains an important contact site for the 11S proteasome activator (PA28) [89]. This particle is a multisubunit regulatory complex that stimulates the endopeptidase activities of the 20S proteasome through association with the α-rings [90]. It is believed that the 11S activator serves to open the exit gate of the proteasome to accelerate substrate throughput [91]. The decrease in α6 following HDAC inhibition may further hinder proteasome substrate processing by depleting the cell of 11S-bound proteasomes.

The identification of the proteasome as a potential target for HDAC inhibitors is interesting from a pharmacological point of view as proteasome inhibitors comprise a new class of therapeutic drugs [92,93]. However, unlike direct proteasome inhibitors (i.e. MG-132), which target the active sites of the proteasome, butyrate down-regulates the expression of proteasomal subunits to impair its activity. The ability for both proteasome and HDAC inhibitors to influence proteasome function, as well as their individual chemotherapeutic potential, suggests that such agents would be excellent candidates for combination therapies. Recently, proteasome inhibitors have been used to sensitize cancer cells to HDAC inhibitor-induced apoptosis. This treatment combination was found to effectively reduce NF-κB activity and synergistically enhance the induction of apoptosis than either inhibitor alone (resulting in ∼70–99% cell death) [94–96].

Although our study primarily focused on colon cancer cells, the decrease in proteasome activity by HDAC inhibitors may occur in other cancerous cell types. The decrease in proteasome activity may also influence other cell cycle regulatory proteins controlled by the ubiquitin-proteasome pathway, such as β-catenin, $p27^{Kip1}$, cyclin B

and p53 [97,98]. The proteasome is also involved in antigen processing. The suppression of proteasome activity following HDAC inhibition may therefore impact the antigen presenting function of the colonic epithelium. Although cancer cells are known to readily undergo growth arrest to lose their malignant phenotype following HDAC inhibition, it is possible that there are negative outcomes to HDAC inhibitor treatments [99]. In this regard, knowing how HDAC inhibitors influence cancer cells physiology should help guide the therapeutic use of these inhibitors, as well as which safety problems may arise from their long-term usage.

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References

[1] Bennett WG, Cerda JJ. Benefits of dietary fiber, Myth or medicine? Postgrad Med 1996;99(2. p. 153–6, 166–8, 171–2 [passim].
[2] Reddy BS. Role of dietary fiber in colon cancer: an overview. Am J Med 1999;106(1A):16S–9S [discussion 50S–51S].
[3] Inan MS, et al. The luminal short-chain fatty acid butyrate modulates NF-kappaB activity in a human colonic epithelial cell line. Gastroenterology 2000;118(4):724–34.
[4] Toscani A, Soprano DR, Soprano KJ. Molecular analysis of sodium butyrate-induced growth arrest. Oncogene Res 1988;3(3):223–38.
[5] Fusunyan RD, et al. Butyrate switches the pattern of chemokine secretion by intestinal epithelial cells through histone acetylation. Mol Med 1999;5(9):631–40.
[6] Scheppach W, et al. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. Gastroenterology 1992;103(1):51–6.
[7] Hague A, et al. Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. Int J Cancer 1993;55(3):498–505.
[8] Thomas GL, et al. Enhanced apoptosis in transformed human lung fibroblasts after exposure to sodium butyrate. In Vitr Cell Dev Biol Anim 1996;32(8):505–13.
[9] Velazquez OC, Rombeau JL. Butyrate potential role in colon cancer prevention and treatment. Adv Exp Med Biol 1997;427:169–81.
[10] Hassig CA, Tong JK, Schreiber SL. Fiber-derived butyrate and the prevention of colon cancer. Chem Biol 1997;4(11):783–9.
[11] Csordas A. Butyrate, aspirin and colorectal cancer. Eur J Cancer Prev 1996;5(4):221–31.
[12] Breuer RI, et al. Rectal irrigation with short-chain fatty acids for distal ulcerative colitis. Preliminary report. Dig Dis Sci 1991;36(2):185–7.
[13] Kanauchi O, et al. Butyrate from bacterial fermentation of germinated barley foodstuff preserves intestinal barrier function in experimental colitis in the rat model. J Gastroenterol Hepatol 1999;14(9):880–8.
[14] Wachtershauser A, Stein J. Rationale for the luminal provision of butyrate in intestinal diseases. Eur J Nutr 2000;39(4):164–71.
[15] Boffa LC, et al. Suppression of histone deacetylation in vivo and in vitro by sodium butyrate. J Biol Chem 1978;253(10):3364–6.
[16] Kawai H, et al. Overexpression of histone deacetylase HDAC1 modulates breast cancer progression by negative regulation of estrogen receptor alpha. Int J Cancer 2003;107(3):353–8.
[17] Choi JH, et al. Expression profile of histone deacetylase 1 in gastric cancer tissues. Jpn J Cancer Res 2001;92(12):1300–4.
[18] Halkidou K, et al. Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate 2004;59(2): 177–89.
[19] Zhu P, et al. Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. Cancer Cell 2004;5(5):455–63.
[20] Atadja P, et al. Molecular and cellular basis for the anti-proliferative effects of the HDAC inhibitor LAQ824. Novartis Found Symp 2004;259:249–66 [discussion 266–8, 285–8].
[21] Kelly WK, et al. Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously. Clin Cancer Res 2003;9(10 Pt 1):3578–88.
[22] Piekarz R, Bates S. A review of depsipeptide and other histone deacetylase inhibitors in clinical trials. Curr Pharm Des 2004;10 (19):2289–98.
[23] Mariadason JM, Corner GA, Augenlicht LH. Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: comparison with trichostatin A, sulindac, and curcumin and implications for chemoprevention of colon cancer. Cancer Res 2000;60(16):4561–72.
[24] Boyes J, et al. Regulation of activity of the transcription factor GATA-1 by acetylation. Nature 1998;396(6711):594–8.
[25] Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell 1997;90(4):595–606.
[26] Nakano K, et al. Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. J Biol Chem 1997;272(35):22199–206.
[27] Braun H, et al. Transcription factor Sp3 is regulated by acetylation. Nucleic Acids Res 2001;29(24):4994–5000.
[28] Segain JP, et al. Butyrate inhibits inflammatory responses through NF-kappaB inhibition: implications for Crohn's disease. Gut 2000;47(3): 397–403.
[29] Wu GD, et al. High-level expression of I kappa B-beta in the surface epithelium of the colon: in vitro evidence for an immunomodulatory role. J Leukoc Biol 1999;66(6):1049–56.
[30] Yin L, Laevsky G, Giardina C. Butyrate suppression of colonocyte NF-kappa B activation and cellular proteasome activity. J Biol Chem 2001;276(48):44641–6.
[31] Luhrs H, et al. Butyrate inhibits NF-kappa B activation in lamina propria macrophages of patients with ulcerative colitis. Scand J Gastroenterol 2002;37(4):458–66.
[32] Greten FR, et al. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 2004;118(3):285–96.
[33] Baldwin Jr AS. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649–83.
[34] Maaser C, et al. Colonic epithelial cells induce endothelial cell expression of ICAM-1 and VCAM-1 by a NF-kappa B-dependent mechanism. Clin Exp Immunol 2001;124(2):208–13.
[35] Shishodia S, Aggarwal BB. Nuclear factor-kappa B: a friend or a foe in cancer? Biochem Pharmacol 2004;68(6):1071–80.
[36] Baeuerle PA. I kappa B–NF-kappaB structures: at the interface of inflammation control. Cell 1998;95(6):729–31.
[37] Baeuerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science 1988;242(4878):540–6.
[38] Gilmore TD, Morin PJ. The I kappa B proteins: members of a multifunctional family. Trends Genet 1993;9(12):427–33.
[39] Hirano F, et al. Alternative splicing variants of I kappa B beta establish differential NF-kappa B signal responsiveness in human cells. Mol Cell Biol 1998;18(5):2596–607.
[40] Haskill S, et al. Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. Cell 1991;65(7):1281–9.
[41] Yamazaki S, Muta T, Takeshige K. A novel I kappa B protein, I kappa B-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappa B in the nuclei. J Biol Chem 2001;276(29):27657–62.

[42] Verma IM, et al. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. Genes Dev 1995;9(22):2723–35.
[43] Place RF, et al. Cytokine stabilization of newly synthesized I(kappa)B-alpha. Biochem Biophys Res Commun 2001;283(4):813–20.
[44] Place RF, Haspeslagh D, Giardina C. Induced stabilization of I kappa B-alpha can facilitate its re-synthesis and prevent sequential degradation. J Cell Physiol 2003;195(3):470–8.
[45] Brown K, et al. Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. Proc Natl Acad Sci USA 1993;90(6):2532–6.
[46] Scott ML, et al. The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms. Genes Dev 1993;7(7A):1266–76.
[47] Sun SC, et al. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science 1993;259(5103):1912–5.
[48] Chen Z, et al. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes Dev 1995;9(13):1586–97.
[49] Yaron A, et al. Identification of the receptor component of the I kappa B alpha-ubiquitin ligase. Nature 1998;396(6711):590–4.
[50] Scherer DC, et al. Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. Proc Natl Acad Sci USA 1995;92(24):11259–63.
[51] Miyamoto S, et al. Tumor necrosis factor alpha-induced phosphorylation of I kappa B alpha is a signal for its degradation but not dissociation from NF-kappa B. Proc Natl Acad Sci USA 1994;91(26):12740–4.
[52] Finco TS, Beg AA, Baldwin Jr AS. Inducible phosphorylation of I kappa B alpha is not sufficient for its dissociation from NF-kappa B and is inhibited by protease inhibitors. Proc Natl Acad Sci USA 1994;91(25):11884–8.
[53] Catley L, et al. NVP-LAQ824 is a potent novel histone deacetylase inhibitor with significant activity against multiple myeloma. Blood 2003;102(7):2615–22.
[54] Rock KL, et al. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 1994;78(5):761–71.
[55] Borenfreund E, Borrero O. In vitro cytotoxicity assays. Potential alternatives to the Draize ocular allergy test. Cell Biol Toxicol 1984;1(1):55–65.
[56] Qin Z, Wang Y, Chasea TN. A caspase-3-like protease is involved in NF-kappaB activation induced by stimulation of *N*-methyl-D-aspartate receptors in rat striatum. Brain Res Mol Brain Res 2000;80(2):111–22.
[57] Chen F, et al. Calpain contributes to silica-induced I kappa B-alpha degradation and nuclear factor-kappa B activation. Arch Biochem Biophys 1997;342(2):383–8.
[58] Miyamoto S, Seufzer BJ, Shumway SD. Novel I kappa B alpha proteolytic pathway in WEHI231 immature B cells. Mol Cell Biol 1998;18(1):19–29.
[59] Han Y, et al. Tumor necrosis factor-alpha-inducible I kappa B alpha proteolysis mediated by cytosolic m-calpain. A mechanism parallel to the ubiquitin-proteasome pathway for nuclear factor-kappa B activation. J Biol Chem 1999;274(2):787–94.
[60] Nemeth ZH, et al. Proteasome inhibitors induce inhibitory kappa B (I kappa B) kinase activation, I kappa B alpha degradation, and nuclear factor kappa B activation in HT-29 cells. Mol Pharmacol 2004;65(2):342–9.
[61] Ito CY, Kazantsev AG, Baldwin Jr AS. Three NF-kappa B sites in the I kappa B-alpha promoter are required for induction of gene expression by TNF alpha. Nucleic Acids Res 1994;22(18):3787–92.
[62] Mitsiades CS, et al. Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. Proc Natl Acad Sci USA 2004;101(2):540–5.
[63] Figueiredo-Pereira ME, Berg KA, Wilk S. A new inhibitor of the chymotrypsin-like activity of the multicatalytic proteinase complex (20S proteasome) induces accumulation of ubiquitin-protein conjugates in a neuronal cell. J Neurochem 1994;63(4):1578–81.
[64] Vinitsky A, et al. Inhibition of the chymotrypsin-like activity of the pituitary multicatalytic proteinase complex. Biochemistry 1992;31(39):9421–8.
[65] Li Z, et al. A single amino acid substitution in a proteasome subunit triggers aggregation of ubiquitinated proteins in stressed neuronal cells. J Neurochem 2004;90(1):19–28.
[66] Wojcik C, DeMartino GN. Analysis of Drosophila 26 S proteasome using RNA interference. J Biol Chem 2002;277(8):6188–97.
[67] Gaczynska M, Rock KL, Goldberg AL. Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. Nature 1993;365(6443):264–7.
[68] Yang Y, et al. Proteasomes are regulated by interferon gamma: implications for antigen processing. Proc Natl Acad Sci USA 1992;89(11):4928–32.
[69] Groll M, et al. A gated channel into the proteasome core particle. Nat Struct Biol 2000;7(11):1062–7.
[70] Richon VM, et al. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci USA 2000;97(18):10014–9.
[71] Blagosklonny MV, et al. Histone deacetylase inhibitors all induce p21 but differentially cause tubulin acetylation, mitotic arrest, and cytotoxicity. Mol Cancer Ther 2002;1(11):937–41.
[72] Ruemmele FM, et al. Butyrate mediates Caco-2 cell apoptosis via up-regulation of pro-apoptotic BAK and inducing caspase-3 mediated cleavage of poly-(ADP-ribose) polymerase (PARP). Cell Death Differ 1999;6(8):729–35.
[73] McBain JA, et al. Apoptotic death in adenocarcinoma cell lines induced by butyrate and other histone deacetylase inhibitors. Biochem Pharmacol 1997;53(9):1357–68.
[74] Luque I, Gelinas C. Rel/NF-kappa B and I kappa B factors in oncogenesis. Semin Cancer Biol 1997;8(2):103–11.
[75] Gilmore TD, et al. Rel/NF-kappa B/I kappa B proteins and cancer. Oncogene 1996;13(7):1367–78.
[76] Neurath MF, et al. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. Nat Med 1996;2(9):998–1004.
[77] Rogler G, et al. Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology 1998;115(2):357–69.
[78] Luhrs H, et al. Cytokine-activated degradation of inhibitory kappa B protein alpha is inhibited by the short-chain fatty acid butyrate. Int J Colorectal Dis 2001;16(4):195–201.
[79] Adam E, et al. Potentiation of tumor necrosis factor-induced NF-kappa B activation by deacetylase inhibitors is associated with a delayed cytoplasmic reappearance of I kappa B alpha. Mol Cell Biol 2003;23(17):6200–9.
[80] Soldatenkov VA, et al. Sodium butyrate induces apoptosis and accumulation of ubiquitinated proteins in human breast carcinoma cells. Cell Death Differ 1998;5(4):307–12.
[81] Chen P, Hochstrasser M. Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly. Cell 1996;86(6):961–72.
[82] Gaczynska M, et al. Proteasome subunits X and Y alter peptidase activities in opposite ways to the interferon-gamma-induced subunits LMP2 and LMP7. J Biol Chem 1996;271(29):17275–80.
[83] Chondrogianni N, et al. Central role of the proteasome in senescence and survival of human fibroblasts: induction of a senescence-like phenotype upon its inhibition and resistance to stress upon its activation. J Biol Chem 2003;278(30):28026–37.
[84] Mannhaupt G, et al. Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. FEBS Lett 1999;450(1–2):27–34.
[85] Xie Y, Varshavsky A. RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. Proc Natl Acad Sci USA 2001;98(6):3056–61.

[86] Wyke SM, Russell ST, Tisdale MJ. Induction of proteasome expression in skeletal muscle is attenuated by inhibitors of NF-kappa B activation. Br J Cancer 2004;91(9):1742–50.
[87] Grune T, Reinheckel T, Davies KJ. Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. J Biol Chem 1996;271(26):15504–9.
[88] Qureshi N, et al. The proteasome as a lipopolysaccharide-binding protein in macrophages: differential effects of proteasome inhibition on lipopolysaccharide-induced signaling events. J Immunol 2003;171(3):1515–25.
[89] Kania MA, et al. The proteasome subunit, C2, contains an important site for binding of the PA28 (11S) activator. Eur J Biochem 1996;236(2):510–6.
[90] Whitby FG, et al. Structural basis for the activation of 20S proteasomes by 11S regulators. Nature 2000;408(6808):115–20.
[91] Hendil KB, Khan S, Tanaka K. Simultaneous binding of PA28 and PA700 activators to 20 S proteasomes. Biochem J 1998;332(Pt 3):749–54.
[92] Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol 1998;8(10):397–403.
[93] Adams J, Palombella VJ, Elliott PJ. Proteasome inhibition: a new strategy in cancer treatment. Invest New Drugs 2000;18(2):109–21.
[94] Denlinger CE, et al. Combined proteasome and histone deacetylase inhibition in non-small cell lung cancer. J Thorac Cardiovasc Surg 2004;127(4):1078–86.
[95] Yu C, et al. The proteasome inhibitor bortezomib interacts synergistically with histone deacetylase inhibitors to induce apoptosis in Bcr/Abl+ cells sensitive and resistant to STI571. Blood 2003;102(10):3765–74.
[96] Pei XY, Dai Y, Grant S. Synergistic induction of oxidative injury and apoptosis in human multiple myeloma cells by the proteasome inhibitor bortezomib and histone deacetylase inhibitors. Clin Cancer Res 2004;10(11):3839–52.
[97] Rolfe M, Chiu MI, Pagano M. The ubiquitin-mediated proteolytic pathway as a therapeutic area. J Mol Med 1997;75(1):5–17.
[98] Hershko A. Roles of ubiquitin-mediated proteolysis in cell cycle control. Curr Opin Cell Biol 1997;9(6):788–99.
[99] Marks PA, et al. Histone deacetylase inhibitors: development as cancer therapy. Novartis Found Symp 2004;259:269–81 [discussion 281–8].