Essential Role of the JAK/STAT1 Signaling Pathway in the Expression of Inducible Nitric-oxide Synthase in Intestinal Epithelial Cells and Its Regulation by Butyrate

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Nitric oxide (NO) is a free radical with important functions in a number of physiological and pathophysiological processes, including inflammation and cancer. It is synthesized from L-arginine by nitric-oxide synthases (NOS),3 which include neuronal NOS, endothelial NOS, and inducible NOS (iNOS). Although neuronal and endothelial NOS are constitutively expressed, the expression of iNOS is regulated at the transcriptional level by proinflammatory cytokines such as TNF, IFNγ, and IL-1 or by hypoxia and LPS (1, 2).

The expression of iNOS has been shown to be elevated frequently in tumors. Tumor-associated fibroblasts and inflammatory cells appear to be the predominant, but not the exclusive, source of NO. The expression of iNOS correlates inversely with tumor grade in brain (3) and breast tumors (4) but not, for example, in thyroid cancer (5). Likewise, there are conflicting reports regarding the relationship between iNOS expression and tumor progression in colon cancer. In humans, the expression of iNOS is reportedly reduced in the earliest neoplastic lesions in the colon, the aberrant crypt foci (6), but iNOS has also been shown to be overexpressed in the azoxymethane-induced colonic aberrant crypt foci (7). Some reports have suggested that in colon cancer the expression of iNOS correlates with VEGF expression and microvascular density, whereas other publications have disputed that finding (8–10).

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The abbreviations used are: NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; HDAC, histone deacetylase; HDACi, inhibitors of HDAC activity; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid; VEGF, vascular endothelial growth factor; IFNγ, interferon γ; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; JAK, Janus kinase; STAT, signal transducers and activators of transcription; IEC, intestinal epithelial cell; iK-Ras, inducible K-Ras; IPTG, isopropyl β-D-galactopyranoside; siRNA, small interfering RNA.

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with wild type p53 but to promote the growth of colon cancer cells with mutant p53 through VEGF activation (16).

The biological activity of NO also depends on its local concentration and on the duration of the exposure of cells to NO. For example, high concentrations of NO (typically produced by iNOS) have been shown to induce apoptosis, whereas low concentrations of NO (produced by endothelial or neuronal NOS) can actually protect cells from apoptosis. However, it appears that exposure to moderate to high concentrations of NO promotes neoplastic transformation both in vitro and in vivo. Consistent with this notion, overexpression of iNOS in a human colon cancer cell line enhances VEGF expression and tumor progression (16), and the inhibition of iNOS in a glioma cell line reduces tumor growth (17). NO has been shown to stimulate angiogenesis not only through its ability to increase proliferation and migration of endothelial cells (18) but also through direct activation of HIF1α, an important positive regulator of VEGF expression (19). Thus, although modulation of NO signaling has been suggested as a novel approach for the treatment of tumors, a better understanding of the role of nitric oxide in tumor biology is required in order to design suitable therapeutic strategies.

In this study we have shown that treatment of intestinal epithelial cells with IFNγ and LPS, or with the combination of TNF and IFNγ, activates the expression of iNOS at the transcriptional level through a JAK/STAT1-dependent pathway. We show that butyrate (a dietary chemopreventive agent), but not other inhibitors of HDAC activity, promotes iNOS expression and NO production in intestinal epithelial cells, identifying a biological activity of butyrate that appears to be independent of its ability to inhibit HDAC activity. In contrast, and consistent with their anti-inflammatory activity, all HDACi inhibited NO production in macrophages and in intestinal myofibroblasts. HDACi are drugs with potent chemopreventive and chemotherapeutic activity. They inhibit tumor development through inhibition of cell proliferation and induction of cell death and differentiation (20). Their ability to modulate NO signaling in both tumor cells and stromal cells is likely to play a significant role in their anti-inflammatory activity and may therefore contribute to their ability to halt tumor progression.

EXPERIMENTAL PROCEDURES

Cell Culture and Cytokine Treatment—Rat intestinal epithelial cells (IEC-6), transfected with an inducible K-RasVal-12 cDNA (IEC-iK-Ras), were a generous gift from Dr. Raymond DuBois (21). Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 400 μg/ml G418 (Invitrogen), and 150 μg/ml hygromycin B (Sigma). Expression of oncogenic Ras was induced by 5 mM IPTG (Calbiochem). iNOS expression was induced by treatment of cells with IFNγ (10 ng/ml) and LPS (10 μg/ml of LPS) (Sigma) or by TNF (10 ng/ml) and LPS (10 μg/ml). RAW cells were grown in RPMI 1640 medium and intestinal myofibroblasts MIC216 in Dulbecco’s modified Eagle’s medium under standard culture conditions (22).

Western Blot Analysis—Western blot analysis was performed using standard procedures. Approximately 50 μg of total cell lysates were fractionated in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were incubated with antibodies for 1 h at room temperature or overnight at 4°C. Chemiluminescence (ECL, Amersham Biosciences) was used for the visualization of immune complexes. Antibodies specific for iNOS, STAT1, acetyl H3, and acetyl H4 were purchased from Cell Signaling Technology (Beverly, MA).

Analysis of NO—NO production was measured by the Griess assay (23). Briefly, cell supernatants (50 μl) were mixed with 50 μl of Griess reagent (Sigma). After 10 min, product formation was determined colorimetrically at 540 nm. The experiments were done in triplicates, repeated at least three times, and the results of a representative experiment are shown.

Transient Transfections and Reporter Gene Assay—A reporter plasmid for the rat iNOS promoter region, containing 2158 bp, was kindly provided by Dr. Mami Takahashi (24). Cells were transfected with 1 μg of plasmid DNA per 12-well plate using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). Transfection efficiency was normalized by cotransfection with pTK-Renilla (dual luciferase reporter assay system, Promega, Madison, WI). Cells were either left untreated or treated with IFNγ/LPS or TNF/IFNγ in the absence or presence of IPTG (5 μM). Basal activity and IFNγ/LPS-inducible transcriptional activity were determined 48 h after transfection (24 h after treatment). Results are expressed as the relative promoter activity, calculated from the ratio between luciferase and Renilla (LUC/REN) activity. In cotransfection experiments, the iNOS promoter construct was transfected together with nontargeting small interfering RNA (siRNA) or siRNA directed against the coding region of STAT1 (Dharmacon, Lafayette, CO). Cells were transfected with 50 nM STAT1 siRNA using the Lipofectamine 2000 method (Invitrogen).

RESULTS

LPS and IFNγ Synergistically Activate the Expression of iNOS —Although stromal cells are the predominant source of NO, epithelial cells have also been shown to express iNOS and to produce NO in response to proinflammatory cytokines such as IL-1 (24). In this study we examined whether another proinflammatory cytokine, IFNγ, is also able to stimulate the expression of iNOS in intestinal epithelial cells and whether it cooperates with LPS, a product of the bacterial cell wall. We performed experiments in rat IECs with IPTG-inducible expression of mutant K-Ras, the IEC-iK-Ras cells (21). We first treated cells with IFNγ alone, LPS alone, or a combination of both agents, which has been shown to be a potent inducer of iNOS expression in macrophages. Unlike in macrophages, neither IFNγ alone nor LPS alone was able to induce the expression of iNOS; however, when intestinal cells were co-treated with both agents, the expression level of iNOS rose significantly (Fig. 1A). Consistently, the level of NO significantly increased only in cells treated with both IFNγ and LPS (Fig. 1B). The induction of NO appeared in a concentration- and time-dependent manner as shown in supplemental Fig. 1, A and B. Type I IFN (IFNβ) failed to synergize with LPS in induction of NO (supplemental Fig. 1C).

The Role of Oncogenic Ras in the Expression of iNOS—Constitutive signaling by Ras has been suggested to induce a proin-
flammatory environment through activation of proteins such as IL-8 and COX-2 (21, 25, 26). To determine whether in intestinal epithelial cells constitutive signaling by oncogenic Ras also affects the production of NO, another proinflammatory mediator, we induced Ras signaling in IEC-iK-Ras cells with 5 mM IPTG for 24 h before treating the cells with IFN/H9253 or LPS. As in the absence of oncogenic Ras, IFN/H9253 or LPS alone failed to induce the expression of iNOS. However, the levels of iNOS produced in response to treatment with LPS/IFN/H9253 in cells with oncogenic Ras were significantly higher than in the absence of Ras signaling (Fig. 1A). Consistent with the expression of iNOS, the levels of NO in supernatants were elevated only in response to the combined treatment of cells with both IFN/H9253 and LPS, and the activation of mutant Ras increased the levels of nitric oxide greatly (Fig. 1B). IFN/H9253 was also able to synergize with TNF in the induction of iNOS (not shown) and in the production of NO (Fig. 1C). In agreement with data shown in Fig. 1A, cells expressing activated Ras produced considerably more NO in response to treatment with TNF/IFN/H9253 (Fig. 1B). These results are consistent with findings that the levels of iNOS expression and NO production are elevated during inflammation and frequently increased in transformed cells.

Next we determined whether IFN/H9253 and LPS regulate the expression of iNOS at the level of transcription. IEC-iK-Ras cells were transfected with the rat iNOS promoter construct

![Image](https://example.com/image1.png)

**FIGURE 1.** Treatment of IECs with IFN/LPS or TNF/IFN induces iNOS expression and NO production. A, cells were left untreated or were treated with IFNγ, LPS, or a combination of both. Cells were treated in the absence or presence of 5 mM IPTG as indicated. The expression of iNOS (A) and production of NO (B) were determined as described under “Experimental Procedures.” C, NO production was determined in the absence or presence of IPTG in response to treatment with LPS/IFN/H9253 or TNF/IFN/H9253. IEC-iK-Ras cells were transfected with the iNOS promoter and were treated with LPS, IFN/H9253, or a combination of both (left) or with LPS/IFN/H9253 in the absence or presence of IPTG as indicated (right). All treatments were for 24 h. CTRL, control.

![Image](https://example.com/image2.png)

**FIGURE 2.** JAK activity is required for the induction of iNOS. A, the expression of iNOS was determined in control cells (lanes 1) and in cells treated with LPS/IFNγ alone (lanes 2) or in the presence of JAK inhibitor 1 (lanes 3). Experiments were performed in the absence or presence of IPTG as indicated. B, the effect of JAK inhibitor (JAKI) on LPS/IFNγ-induced production of NO in the absence or the presence of IPTG. CTRL, control.
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FIGURE 3. STAT1 is required for the induction of iNOS and for NO production in response to LPS/IFN or TNF/IFN. A, IECs were transfected with nontargeting siRNA or with STAT1 siRNA and treated with LPS, IFN γ, or a combination of both for 24 h as indicated. The levels of STAT1, iNOS, and IRF1 were determined by immunoblotting. NSB, nonspecific band. B, the amount of NO produced in response to LPS/IFN γ or TNF/IFN γ was determined in cells transfected with nontargeting siRNA (NSP) or siRNA specific for STAT1 as indicated. C, IECs were transfected with the rat iNOS promoter in the presence of nontargeting siRNA (nonspecific (NSP)) or STAT1 siRNA. At 24 h after transfection, cells were treated with LPS/IFN γ or TNF/IFN γ for 24 h. CTRL, control.

JAK activity (JAK inhibitor 1, Calbiochem) for 2 h before stimulation with IFN/ LPS. As shown in Fig. 2, inhibition of JAKs completely prevented the induction of iNOS in response to IFN/LPS (Fig. 2A) and consistently inhibited the production of NO (Fig. 2B).

To determine whether STAT1 is required for the induction of iNOS in response to IFN/LPS treatment, we silenced STAT1 expression through siRNA, using oligonucleotides that specifically target rat STAT1. As shown in Fig. 3A, we successfully silenced STAT1 expression. Consistent with our published data (27), STAT1 deficiency strongly interfered with the induction of iNOS in response to IFN/LPS treatment, demonstrating that STAT1 mediates the synergism between LPS and IFN γ (Fig. 3A). However, our data also revealed that the expression of STAT1 is not sufficient for the induction of iNOS, as the treatment of cells with IFN γ alone, which resulted in a robust expression of NO (Fig. 2A), failed to induce iNOS expression (Fig. 3A). Consistent with impaired iNOS expression in STAT1-deficient cells, these cells also produced significantly lower amounts of NO in response to treatment with IFN/ LPS or TNF/IFN γ (Fig. 3B).

To establish whether STAT1 is required for transcriptional induction of iNOS by LPS/IFN γ or TNF/IFN γ, we transfected cells with the iNOS promoter in the presence of nontargeting siRNA or siRNA specific for STAT1. At 24 h after transfection, cells were either left untreated or were treated for 24 h with LPS/IFN γ or TNF/IFN γ. As shown in Fig. 3C, silencing of STAT1 did not interfere with the basal activity of iNOS, but it markedly lowered the inducibility of the iNOS promoter in response to treatment with LPS/IFN γ or TNF/IFN γ. These data demonstrate that STAT1 is required for the transcriptional induction of iNOS in response to IFN/LPS and TNF/LPS. Together, these data establish the requirement for the JAK/STAT1 signaling pathway for the expression of iNOS and for the production of NO in intestinal epithelial cells.

Butyrate, but Not Other HDAC Inhibitors, Enhances NO Production in Intestinal Epithelial Cells—We have recently shown that IFN γ regulates the expression of a subset of its target genes in an HDAC-dependent manner (27). We therefore examined whether the expression of iNOS and the production of NO in

(24) and were treated with IFN γ or LPS individually or with a combination of both. As shown in Fig. 1D (left panel), neither IFN γ nor LPS alone was able to stimulate the activity of the iNOS promoter; however, treatment of cells with LPS/IFN γ increased iNOS promoter activity, demonstrating that LPS/IFN γ synergize at the level of transcription. In addition, induction of the activated Ras by IPTG increased transcriptional activity of the iNOS promoter (Fig. 1D, right panel), demonstrating that Ras signaling augments the expression of iNOS in response to IFN γ/LPS treatment at the level of transcription.

JAK/STAT1 Signaling Is Required for iNOS Expression and NO Production—Despite the fact that STAT1 is a major transcription factor in signaling by IFN γ, we and others have shown that IFN γ can signal through both STAT1-dependent and -independent pathways (27–30). To determine whether the activity of JAKs, kinases that phosphorylate STAT1, is required for the induction of iNOS and NO expression in response to IFN γ/LPS, we pretreated cells with a pharmacological inhibitor of
response to IFN/LPS are regulated by inhibitors of HDAC activity, which act as important anti-inflammatory and antitumorigenic compounds. Cells were treated with LPS/IFNγ in the absence or presence of four structurally unrelated HDACi: butyrate, SAHA, TSA, and apicidin. As shown in Fig. 4A, butyrate, but not the other HDAC inhibitors, significantly enhanced the expression of iNOS and the production of NO in response to IFN/LPS. We repeated the experiments four times and obtained similar results. Butyrate, but not SAHA, TSA, or apicidin, also increased the expression of iNOS and the production of NO in the presence of mutant Ras (Fig. 4B). Consistently, butyrate, but not other HDAC inhibitors, increased the production of NO also in response to treatment of epithelial cells with TNF/IFNγ (data not shown).

Butyrate displays anti-inflammatory properties, and therefore its ability to increase the production of NO was a surprising result. We expanded our experiments and examined the regulation of NO production by HDAC inhibitors in two stromal cell types, macrophages and intestinal myofibroblasts. In contrast to intestinal epithelial cells, all HDACi decreased the production of NO in macrophages (Fig. 5A) and intestinal myofibroblasts (Fig. 5B) in response to treatment with IFNγ/LPS or IFNγ/TNF, consistent with their anti-inflammatory activity. Because butyrate displayed a unique biological activity in intestinal epithelial cells, we examined the possibility that the four HDACi that we used differ in their ability to induce acetylation of histones in these cells. We treated cells with IFNγ/LPS in the absence or presence of butyrate, SAHA, TSA, or apicidin. Consistent with data shown in Fig. 4, only butyrate was able to promote the production of NO in response to IFN/LPS (Fig. 5C). However, all HDAC inhibitors potently induced acetylation of both histones H3 and H4 in intestinal epithelial cells (Fig. 5D), excluding the possibility that the unique biological activity of butyrate is due to its differential ability to inhibit HDAC in intestinal epithelial cells.

These data therefore demonstrate that butyrate augments iNOS expression and NO production in intestinal epithelial cells independently of its ability to inhibit HDAC activity. In contrast, its ability to inhibit NO production in macrophages and myofibroblasts is shared by other inhibitors of HDAC activity.

**The Role of NO in Survival of Intestinal Epithelial Cells**—Because NO is an important regulator of cell survival, we examined the possibility that butyrate, which exerts its chemopreventive activity mainly through induction of cell cycle arrest and apoptosis, modulates these processes, at least in part, through its ability to enhance the production of NO. Cells were treated with a combination of LPS/IFN alone or in the presence of butyrate for 24 h, and cell viability was compared in the absence or presence of IPTG (Fig. 6). As shown in Fig. 6A, treatment of cells with IFN/LPS induced apoptosis preferentially in the absence of signaling by oncogenic Ras. Significantly, butyrate completely prevented apoptosis induced by LPS/IFN in the absence of Ras signaling, but it enhanced IFN/LPS-mediated apoptosis upon induction of Ras signaling by the addition of IPTG (Fig. 6, A

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**Figure 4.** Butyrate, but not other HDAC inhibitors, enhances iNOS expression and NO production in intestinal cells. Cells were left untreated (ctrl) or were treated with IFNγ/LPS alone or in the presence of 3 mM butyrate (Bu), SAHA (0.5 or 3 μM), TSA (0.1 or 1 μM), or apicidin (0.2 or 1 μM) in the absence (A) or presence (B) of IPTG for 24 h. The amount of NO (upper panels) and the expression of iNOS (lower panels) were determined as described under “Experimental Procedures.”

**Figure 5.** HDAC inhibitors interfere with NO production in macrophages (A) and intestinal myofibroblasts (B), but not in intestinal epithelial cells (C), and induce acetylation of histones H3 and H4 (D). A–C, treatments were as indicated, and NO was measured as described under “Experimental Procedures.” D, the amount of acetylated (ac) H3 and acetylated H4 was determined by immunoblotting using antibodies specifically recognizing acetylated histones H3 or H4. β-Actin was used to control for equal loading (ctrl). Bu, butyrate; AP, apicidin.
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**A**

**B**

**C**

**FIGURE 6.** Butyrate acts as a survival factor in nontransformed epithelial cells but accelerates apoptosis in cells with oncogenic Ras. **A,** cells were treated with IFN/LPS in the absence or presence of butyrate (Bu), as indicated, for 24 h. B, the amount of apoptosis in cells treated with IFN/LPS in the absence or presence of HDACi was determined by propidium iodide staining. C, the mitochondrial membrane potential was determined 24 h after treatment of cells with IFN/LPS or IFN/LPS/butyrate. CTRL, control; APC, apicidin.

and B). Consistent with this, we showed that in the absence of IPTG butyrate prevented IFN/LPS-induced collapse in the mitochondrial membrane potential but accelerated the decrease of mitochondrial membrane potential in cells in which constitutive Ras signaling was induced by IPTG (Fig. 6C). Thus, although butyrate increased NO production in both nontransformed and transformed intestinal epithelial cells, it acted as a survival factor in nontransformed cells but induced apoptosis in cells transformed with oncogenic Ras. These data therefore strongly suggest that 1) NO is not a major regulator of apoptosis in these cells and 2) also that butyrate does not regulate cell survival through NO production. In addition, the fact that SAHA, TSA, and apicidin, three HDACi that failed to increase NO production, were also potent inhibitors of LPS/IFN-induced cell death (Fig. 6B) also argue against the role of NO in HDACi-induced apoptosis.

Finally, these data also suggest that treatment of cells with IFN/LPS does not regulate cell viability though induction of NO. Indeed, although we have demonstrated that treatment of IEC-1-K-Ras cells with an NO donor, S-nitroso-N-acetylpenicillamine (SNAP), like LPS/IFNγ, can induce cell death (supplemental Fig. 2A), pharmacological inhibition of iNOS by a selective inhibitor 1400W (supplemental Fig. 2B) did not restore the viability upon treatment of cells with LPS/IFNγ, as measured by the dissipation of the mitochondrial membrane potential (supplemental Fig. 2A) or by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (supplemental Fig. 2C). Consistently, a scavenger of NO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) did not restore the viability of cells treated with LPS/IFNγ (supplemental Fig. 2C).

**DISCUSSION**

Proinflammatory stimuli elicit the expression of iNOS and COX-2, two proteins that play a significant role in the inflammatory response, and thereby contribute to the development of tumors at sites of chronic inflammation. Although the expression of COX-2 and iNOS occurs predominantly in stromal cells, such as macrophages and fibroblasts, epithelial cells have also been shown to respond to certain stimuli with induction of both iNOS and COX-2 (21, 24). Recently, physical interaction between COX-2 and iNOS has been shown to result in enhanced COX-2 catalytic activity (31), establishing a mechanism for the molecular synergy between major mediators of inflammation.

In this study we have shown that treatment of intestinal epithelial cells with LPS/IFNγ or TNF/IFNγ, but not with any one of these stimuli individually, induces the expression of iNOS at the level of transcription. The synergism between LPS and IFNγ is not due to the elevated levels of TLR4 or IFNγ receptors in cells treated with both agents (data not shown). Instead, we show that synergism between IFNγ and LPS occurs at the transcriptional level and that it requires STAT1. Further, we demonstrate that during Ras-induced transformation of epithelial cells the expression of iNOS and the production of NO are markedly increased. This is consistent with the report that stable transfection of rat intestinal epithelial cells with K-Ras Asp-12 resulted in increased IL-1- and LPS-mediated iNOS expression (24). It is also consistent with our preliminary findings that primary colon tumors have increased expression of iNOS compared with the normal adjacent tissue. Consistently, N-Ras and B-Raf mutations in human melanoma have been shown to drive constitutive iNOS expression and NO production, which contribute to poor patient survival (32).

The regulation of iNOS expression is the main regulatory step in controlling the activity of iNOS. The promoter region of the rat iNOS promoter contains adjacent binding sites for NF-κB, AP1, STAT1, and IRF1 (33). Although NF-κB has been shown to be a major transcription factor responsible for the induction of iNOS in tumor cells, we present data demonstrating that in intestinal epithelial cells intact JAK/STAT1 signaling is required for the induction of iNOS and the production of NO in response to both LPS/IFNγ and TNF/IFNγ. Consistent with our findings, tumor-associated macrophages isolated from STAT1-deficient mice fail to express iNOS and to produce NO (34). We demonstrated that STAT1 deficiency significantly impairs transcriptional activation of the iNOS promoter.

4 L. Klampfer, unpublished results.
by both LPS/IFNγ and TNF/IFNγ. Although our data clearly have established that STAT1 is required for the induction of iNOS, they also reveal that STAT1 is not sufficient for the induction of iNOS, as treatment of cells with IFNγ alone, which is sufficient for optimal STAT1 expression, did not result in iNOS expression. Thus, although our data demonstrate that STAT1 is required for the synergism between LPS and IFNγ, they also show that STAT1 must cooperate with other transcription factors that regulate the iNOS promoter (33, 35, 36). Experiments to elucidate the complex nature of the interplay of STAT1 with other transcription factors that are induced by IFNγ and LPS (and activated in response to Ras signaling) are under way in our laboratory.

HDAC inhibitors have potent anti-inflammatory activity both in vitro and in vivo. Their capacity to inhibit proliferation and to modulate the activity of proinflammatory cytokines is fundamental for their ability to curb inflammation. In addition, TSA and SAHA have been shown to enhance LPS-induced expression of NO in microglial cells (37); and TSA, but not butyrate, induces iNOS promoter activity in the human colon cancer cell line DLD-1 (38). Butyrate enemas have been shown to alleviate ulcerative colitis, a condition in which iNOS and NO are overproduced (39, 40), and butyrate has been shown to improve the efficacy of treatment with 5-Asa (41). We showed that, consistent with their anti-inflammatory activity, HDAC inhibitors significantly reduced NO production in macrophages and in intestinal myofibroblasts. In contrast, we showed that in intestinal epithelial cells butyrate actually increased the production of NO. Interestingly, in intestinal cells butyrate appeared to modulate NO signaling independently of its ability to inhibit HDAC activity, as other inhibitors of HDAC activity that we tested failed to augment NO production in response to treatment with IFNγ/LPS or IFNγ/TNF.

The role of NO overproduction in the intestinal epithelium is not completely understood. In addition to a detrimental role of the sustained presence of NO in acute colitis, NO has also been shown to be instrumental in maintaining the integrity of the gastric mucosa. For example, NO donors have been shown to protect from nonsteroidal anti-inflammatory drug-induced ulcers (42) and the induction of iNOS has been shown to protect against intestinal injury in a model of induced colitis (43). It is therefore possible that the ability of butyrate to increase the production of NO in intestinal cells in response to inflammatory stimuli actually contributes to the protection of gastric mucosa offered by this short chain fatty acid. In addition, the ability of butyrate to regulate NO signaling is likely to contribute to its chemopreventive and chemotherapeutic activity. However, the biological significance of the increased production of NO in intestinal epithelial cells and the pathways whereby butyrate regulates NO signaling remain to be determined.

We have shown here that although butyrate increases NO production in the absence or presence of the mutant Ras, it acts as a survival factor in nontransformed cells while inducing apoptosis in cells with activated Ras. These results are consistent with a divergent role of butyrate in vivo. It has been shown that normal epithelial cells proliferate and transformed cells undergo apoptosis in response to butyrate (44), and we have implicated the role of oncogenic Ras in response to butyrate before (45). However, our results argue against the role of NO in butyrate-induced cell death and demonstrate that there is no simple relationship between cell survival and NO in intestinal epithelial cells.

It is likely that the biological activity of NO differs in nontransformed cells and in cells with constitutive Ras signaling. It has been reported that NO induces apoptosis selectively in Ras-transformed fibroblasts (46). Consistently, our preliminary data suggest that NO donors induce apoptosis preferentially in epithelial cells with mutant Ras. In addition, enhanced expression of iNOS in cells that harbor activated Ras may turn out to play an important role in vivo in processes such as cell migration and angiogenesis. Indeed, overexpression of iNOS in cells that harbor activated Ras may turn out to play an important role in vivo in processes such as cell migration and angiogenesis. Indeed, overexpression of iNOS in cells that harbor activated Ras may turn out to play an important role in vivo in processes such as cell migration and angiogenesis. Indeed, overexpression of iNOS in cells that harbor activated Ras may turn out to play an important role in vivo in processes such as cell migration and angiogenesis.

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