Regulation of PTEN Expression in Intestinal Epithelial Cells by c-Jun NH2-Terminal Kinase Activation and Nuclear Factor-κB Inhibition

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Abstract

The tumor suppressor protein phosphatase and tensin homologue deleted on chromosome ten (PTEN) plays an important role in intestinal cell proliferation and differentiation and tumor suppression by antagonizing phosphatidylinositol 3-kinase. Despite its importance, the molecular mechanisms regulating PTEN expression are largely undefined. Here, we show that treatment of the colon cancer cell line HT29 with the differentiating agent sodium butyrate (NaBT) increased PTEN protein and mRNA expression and induced c-Jun NH2-terminal kinase (JNK) activation. Inhibition of JNK by chemical or genetic methods attenuated NaBT-induced PTEN expression. In addition, our findings showed a cross-talk between nuclear factor κB (NF-κB) and JNK with respect to PTEN regulation. Overexpression of the NF-κB superrepressor increased PTEN expression and JNK activity, whereas overexpression of the p65 NF-κB subunit reduced both basal and NaBT-mediated JNK activation and PTEN expression. Moreover, we showed that overexpression of PTEN or treatment with NaBT increased expression of the cyclin-dependent kinase inhibitor p27kip1 in HT29 cells; this induction was attenuated by inhibition of PTEN or JNK expression or overexpression of p65. Finally, we show a role for PTEN in NaBT-mediated cell death and differentiation. Our findings suggest that the JNK/PTEN and NF-κB/PTEN pathways play a critical role in normal intestinal homeostasis and colon carcinogenesis. [Cancer Res 2007;67(16):7773–81]

Introduction

The tumor suppressor protein phosphatase and tensin homologue deleted on chromosome ten (PTEN) antagonizes the activity of phosphatidylinositol 3-kinase (PI3K) by dephosphorylating the D3-phosphate group of lipid second messengers, thus serving as a negative regulator of the PI3K pathway (1). PTEN inhibits downstream functions mediated by the PI3K pathway, such as cell growth and survival, cell migration, and invasion (2), and cell cycle progression through the regulation of the expression of the cyclin-dependent kinase inhibitor protein p27kip1 (3), which is induced by PTEN in various cells (4, 5). Previously, we showed that inhibition of PI3K or overexpression of PTEN significantly enhances intestinal cell differentiation either spontaneously or induced by the short-chain fatty acid sodium butyrate (NaBT; ref. 6), a histone deacetylase inhibitor produced in the colon by breakdown of dietary fiber (7). PTEN expression correlates with expression of Cdx-2, a homeodomain protein required for intestinal epithelial cell differentiation, along the length of the murine colon (8). Moreover, PTEN stimulates Cdx-2 protein expression and the transcriptional activity of the Cdx-2 promoter, thus further indicating a role for PTEN in the process of intestinal differentiation. Despite the importance of PTEN in apoptosis and differentiation, little is known about the regulation of PTEN expression.

Nuclear factor κB (NF-κB) is a heterodimer consisting of the DNA binding subunit p50 and the transactivation subunit RelA/p65. The activation pathway of NF-κB is regulated by an endogenous cytoplasmic inhibitor, IκB, which, in response to certain stimuli, is phosphorylated and degraded, leaving NF-κB to translocate into the nucleus (9). NF-κB is a central regulator of the transcriptional activation of a number of genes involved in apoptosis, differentiation, and growth; induction of these genes in intestinal epithelial cells by activated NF-κB profoundly influences mucosal inflammation, repair, and inflammation-associated gastrointestinal cancers (10, 11). Recently, we have shown a novel feedback regulation of PTEN through tumor necrosis factor α (TNFα)-mediated NF-κB activation (12). In agreement with our findings, Vasudevan et al. (13) reported a suppressive effect of NF-κB activation on PTEN expression and the prevention of apoptosis. Given the roles of PTEN in antagonizing PI3K-mediated cell survival and tumorigenesis, these findings suggest that PTEN plays an important role in NF-κB function.

c-Jun NH2-terminal protein kinase (JNK) is a subfamily of the mitogen-activated protein kinase superfamily (14). JNK has three isoforms (JNK1, JNK2, and JNK3). Among them, JNK1 and JNK2 are ubiquitously expressed whereas JNK3 is mainly expressed in neuronal tissues and in the heart (15). JNK was originally identified by its ability to specifically phosphorylate the transcription factor c-Jun on its NH2-terminal transactivation domain. The JNK pathway and NF-κB play important roles in numerous physiologic processes (16). For example, the balance between NF-κB and JNK activity controls dendritic cell survival (17). JNK inhibition results in NF-κB activation in multiple myeloma cell lines (18), and NF-κB activation induces MUC2 transcription whereas JNK activation inhibits this induction in human colon epithelial cells (19). Previously, we found that induction of intestinal cell differentiation is associated with increased JNK activity and c-Jun phosphorylation (20). In agreement with these findings, inhibition of JNK has been shown to attenuate intestinal cell differentiation (21).

The purpose of our present study was to determine the role of JNK in the regulation of PTEN expression. Here, we show that NaBT induces NF-κB inhibition and JNK activation, leading to PTEN expression in intestinal cells. Interestingly, our findings show a cross-talk mechanism between the NF-κB and JNK pathways on PTEN regulation. Moreover, NaBT induce p27kip1 expression through the JNK/PTEN and NF-κB/PTEN pathways. Our results...
identify PTEN as a downstream target of the JNK pathway. In addition, our findings suggest that the JNK/PTEN signaling pathway may regulate intestinal cell differentiation through the regulation of p27Kip1 expression.

Materials and Methods

Materials. NaBt and c-Jun protein were purchased from Sigma Chemical Company. SP600125 was from Calbiochem. Mouse anti-human PTEN monoclonal antibody, rabbit anti-h-c-Jun polyclonal antibody, rabbit anti-p50 polyclonal antibody, rabbit anti-p65 polyclonal antibody, rabbit anti-extracellular signal-regulated kinase (ERK)-1 polyclonal antibody, and rabbit anti-JNK1 and anti-JNK2 antibodies were obtained from Santa Cruz Biotechnology. Mouse anti-human JNK1/JNK2 antibody was from BD PharMingen. Rabbit anti-β-actin antibody was from Sigma, JNK1, JNK2, and non-targeting control siRNA SMARTpool were purchased from Dharmacon, Inc. The SMARTpool for targeting JNK1 consisted of four pooled SMARTSelection-designed siRNAs: JNK1 siRNA1 (5'-UCACAGUGCGUAAAC-GAUA-3'), JNK1 siRNA2 (5'-GAUUGGAGAUCACAUU-3'), JNK1 siRNA3 (5'-AAAGUAAACCGCAACAUU-3'), and JNK1 siRNA4 (5'-GAAGAAGACCU-GACAA-3'). The SMARTpool for targeting JNK2 consisted of four pooled SMARTSelection-designed siRNAs: JNK2 siRNA1 (5'-GGAAGAGC-CAAUUAACAA-3'), JNK2 siRNA2 (5'-AAAGAGACUUAAGCCGAA-3'), JNK2 siRNA3 (5'-CAAAAGGUUUGUUGUA-3'), and JNK2 siRNA4 (5'-GAUGAAGGUAGAAUAUG-3'). All siRNAs were synthesized with UU instead of AA in the 3' overhang on each strand. Adenovirus vectors encoding β-gal (AdCA-LacZ; control) and PTEN (AdCA-PTEN) were from Dr. Akira Hori (Tohoku University School of Medicine, Sendai, Japan). Adenovirus vector encoding hemagglutinin-tagged IκB-α superrepressor (Ad5IkB-α) and its control vector (Ad5GFP) were gifts from Dr. Christian Jobin (University of North Carolina, Chapel Hill, NC). The adenovirus vector encoding NF-κB p65 subunit (Ad5p65) and its control vector (Ad5GFP) were from Dr. Craig Logsdon (MD. Anderson Cancer Center, Houston, TX). [γ-32P]ATP (3,000 Ci/mmole) was from Amersham Pharmacia Biotech. Total RNA was isolated using Ultraspec RNA reagent according to the manufacturer's recommended protocol and as we have previously described (25). Total RNA (5 μg) was reverse transcribed with M-MLV reverse transcriptase and PCR reagents were obtained from Life Technologies, Inc. All other reagents were of molecular biology grade and purchased from Sigma.

Cell culture and treatments. The human colon cancer cell lines HT29 and Caco-2 and human embryonic kidney cell line HEK293 were purchased from American Type Culture Collection (Rockville, MA). Rat intestinal epithelial (RIE-1) cells were obtained from Dr. Kenneth D. Brown (Cambridge Research Station, Babraham, Cambridge, United Kingdom). HT29 cells were maintained in McCoy's 5A supplemented with 10% FCS. Caco-2 cells were incubated in MEM supplemented with 15% fetal bovine serum. HEK293 and RIE-1 cells were cultured in DMEM supplemented with 10% FCS. Cells were infected with adeno vectors at 1 plaque-forming units/cell as previously described (22) and incubated for 24 h before initiating treatment. JNK inhibitor, SP600125, was initially dissolved in DMSO, and effects were compared with cells treated with DMSO at the same final concentration.

Transient transfection and luciferase assays. The JNK1, JNK2, and nontargeting control siRNA SMARTpool duplexes were introduced into cells by electroporation (Gene Pulser, Bio-Rad) as we have previously described (23). siRNA SMARTpool, consisting of four siRNA duplexes, was designed using an algorithm composed of 33 criteria and variables that effectively eliminate nonfunctional siRNA (24). For luciferase assay, HT29 cells were seeded at 1 × 10^6 into 24-well plates in triplicate 24 h before transfection. Cells were then transiently transfected with 0.5 μg of NF-κB reporter plasmid and 0.05 μg of Renilla reporter plasmid to normalize for variation in transfection efficiency, using LipofectAMINE Plus transfection agent following the manufacturer's recommended protocol. Cells were harvested for measurement of firefly and Renilla luciferase activities using the dual luciferase assay system. Firefly luciferase activity was determined by subtracting background signal and normalized to the Renilla activity.

JNK activity assay. Cell lysates were incubated with 1.5 μg of anti-JNK1 or anti-JNK2 antibody overnight at 4°C. Immune complexes were recovered with protein A-Sepharose beads, then washed twice with lysis buffer and once with kinase buffer. Pellets were resuspended in 40 μL of kinase buffer [25 mmol/L Tris (pH 7.4), 2 mmol/L DTT, 0.1 mmol/L Na3VO4, 10 mmol/L MgCl2, and 5 μCi of [γ-32P]ATP] containing c-Jun protein at 30°C for 30 min as previously described (20). The kinase reaction was terminated by addition of SDS sample loading buffer [50 mmol/L Tris (pH 6.8), 100 mmol/L DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol]. The samples were then heated to 95°C for 5 min and resolved by SDS-PAGE. The gels were dried and the phosphorylated protein was visualized by autoradiography.

RNA isolation, RT-PCR, and Northern blot analysis. RNA was isolated from cells using Ultraspec RNA reagent according to the manufacturer's protocol and as we have previously described (25). Total RNA (5 μg) was reverse transcribed with Maloney murine leukemia virus reverse transcriptase and PCR reagents were obtained from Life Technologies, Inc. All other reagents were of molecular biology grade and purchased from Sigma.
NaBT induces PTEN expression in HT29 cells. Treatment with NaBT induces intestinal cell differentiation, whereas overexpression of PTEN enhances this process (6). To better delineate upstream signaling pathways responsible for PTEN induction, we used the human colon cancer cell line HT29, which has wild-type PTEN. Using NaBT, we investigated the role of JNK in PTEN induction. Western blots were performed to determine PTEN protein levels. The blots were probed with anti-PTEN and anti-β-actin antibodies. Densitometric analyses were conducted to quantify PTEN expression. The expression of PTEN was assessed in cells treated with NaBT for 4, 8, or 24 h. The results showed a significant increase in PTEN expression, with the highest levels observed at 24 h.

**Results**

NaBT increases PTEN expression in HT29 cells. Treatment with NaBT induces intestinal cell differentiation, whereas overexpression of PTEN enhances this process (6). To better delineate upstream signaling pathways responsible for PTEN induction, we used the human colon cancer cell line HT29, which has wild-type (i.e., nonmutated) PTEN (30). HT29 is used extensively as a model system for studies on cancer. To investigate the role of JNK in PTEN induction, we used Western blotting and densitometric analysis. The results showed a significant increase in PTEN expression, with the highest levels observed at 24 h.
of intestinal epithelial cell proliferation and differentiation (31). Cells were treated with NaBT (5 mmol/L) for various times and protein was extracted. Western blotting showed a NaBT-mediated increase in PTEN expression, which was apparent by 8 h after treatment and continued for the 48-h time course (Fig. 1A). To examine whether the induction of PTEN protein was associated with an increase in mRNA expression, HT29 cells were treated with NaBT (5 mmol/L) over a time course, and total cellular RNA was extracted for analysis of PTEN expression by Northern blot. As shown in Fig. 1B, NaBT increased PTEN mRNA levels compared with vehicle control, which was apparent by 4 h after treatment and continued for the 48-h time course. Together, these results identify induction of PTEN mRNA and protein levels with NaBT treatment. In spite of the increased mRNA and protein levels in NaBT-treated cells, a time-dependent decrease of PTEN protein levels was noted in control cells, which we speculate may be due to decreased serum concentration over the incubation period (32–34).

To determine whether this induction of PTEN is limited to HT29 cells or occurs in other cells, human colon cancer cell line Caco-2, normal rat intestinal epithelial cell line RIE-1, and human embryonic kidney cell line HEK293 were incubated in the presence or absence of NaBT for 24 h (Fig. 1C). Induction of PTEN protein expression was noted in all cells. These results confirm our findings in HT29 cells and, moreover, suggest a general regulation of PTEN expression by NaBT.

Inhibition of JNK attenuates PTEN induction by NaBT. Previously, we have shown that treatment with NaBT increased JNK activity and c-Jun phosphorylation, which was associated with enterocyte-like differentiation in Caco-2 intestinal cells (6, 20). In agreement with our results, Orchel et al. (21) have shown that inhibition of JNK attenuated NaBT-induced differentiation in HT29 cells. To investigate the possible regulatory effect of JNK on PTEN expression, HT29 cells were pretreated with a selective JNK inhibitor, SP600125 (35), followed by treatment with NaBT for 4, 8, or 24 h as early as 4 h after treatment with maximal induction at 24 h. As expected, NaBT increased PTEN protein expression (Fig. 2A) and mRNA expression (Fig. 2B); this induction was dramatically attenuated by pretreatment with SP600125. These results suggest that NaBT-induced PTEN expression requires JNK activation. Previously, we showed that NaBT increased JNK1

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**Figure 3.** NaBT regulates NF-κB binding activity and overexpression of the p65 NF-κB subunit blocks NaBT-induced PTEN expression. A, HT29 cells were treated with NaBT over a time course. Nuclear protein was extracted and EMSA was done to assess NF-κB binding activity. B, HT29 cells were treated with NaBT for 24 or 48 h. Cytosolic and nuclear proteins were extracted and p65, p50, and β-Actin levels were detected by Western blotting. β-Actin and ERK1 were reprobed as loading control for the cytosol and nuclear fractions, respectively. C, HT29 cells were infected with an adenovirus encoding the NF-κB superrepressor IκB-AA or with control adenovirus encoding GFP. After incubation for 24 h, cells were treated with NaBT for an additional 24 h. Total protein was isolated and Western blot was done for analysis of PTEN protein expression and hemagglutinin (HA)-tagged IκB-AA. D, HT29 cells were infected with an adenovirus encoding the p65 NF-κB subunit or control adenovirus encoding GFP. After incubation for 24 h, cells were treated with NaBT for an additional 24 h. Total protein was isolated and Western blot was done for analysis of PTEN protein expression and p65 NF-κB subunit protein expression. E, HT29 cells were infected with adenovirus constructs and treated with NaBT as described above. Nuclear protein was extracted and EMSA was done; increased NF-κB binding activity by overexpression of the p65 NF-κB subunit was confirmed. PTEN signals from three separate experiments were quantitated densitometrically and expressed as fold change with respect to β-actin.
activity at 8 h and sustained for 72 h after treatment in Caco-2 cells. To determine if NaBT increases JNK1 and JNK2 activity, HT29 cells were treated with NaBT and then harvested for in vitro kinase assays using glutathione S-transferase (GST)-c-Jun as substrate (Fig. 2C). Increased JNK activity was noted for both JNK1 and JNK2 over the time course (4–48 h).

To further show the role of JNK in NaBT-mediated PTEN induction, we transfected cells with siRNA directed to either JNK1 or JNK2 to suppress JNK1 and JNK2 expression, respectively. First, the efficacy of JNK siRNA inhibition was analyzed. JNK1 and JNK2 siRNA expression was decreased by ~30% at 12 h after transfection with JNK1 or JNK2 siRNA, respectively, and a 65% decrease was noted 24 h after transfection (Fig. 2D, left). Transfection with siRNA directed to JNK1 or JNK2 did not affect expression of JNK2 or JNK1, respectively. Based on these results, we then treated cells with NaBT 24 h after transfection with siRNA. Suppression of JNK attenuated NaBT-mediated PTEN induction in HT29 cells as compared with cells transfected with nontargeting control siRNA (Fig. 2D, middle and right). Immunoblotting analysis confirmed that expression of JNK1 or JNK2 was significantly inhibited by JNK1 or JNK2 siRNA for 48 h after transfection. Increased JNK expression was noted with NaBT treatment. Transfection of cells with nontargeting control siRNA had no effect on JNK expression (data not shown). Together, our data indicate that JNK1 and JNK2 are important regulators of PTEN expression.

NF-κB regulation of NaBT-mediated PTEN expression in HT29 cells. We have previously shown an important role for NF-κB in PTEN regulation (12). We next determined the effect of NaBT on NF-κB activity. Cells were treated with a single dose of NaBT (5 mmol/L) and analyzed over a time course; nuclear protein was extracted and analyzed by electrophoretic mobility shift assay (EMSA). Treatment with NaBT increased PTEN mRNA expression from 2 to 48 h after treatment; however, results from EMSA showed no change in NF-κB binding activity from 2 to 8 h after NaBT treatment (Fig. 3A, left) and inhibition of NF-κB binding activity after 24 h (Fig. 3A, right). The specificity of DNA binding was confirmed by competition assays using unlabeled probe in molar excess. To further assess the mechanisms involved in NaBT-mediated inhibition of NF-κB binding activity, HT29 cells were treated with NaBT for 24 or 48 h; cytosolic and nuclear protein were extracted and p65, p50, and IκBα expression was detected by Western blotting. As shown in Fig. 3B, the decreased level of p50 expression was noted in the cytosolic and nuclear fractions with no change in either of p65 or IκBα expression. These data indicate that NaBT inhibition of NF-κB binding activity may be due to decreased levels of nuclear p50.

To assess the involvement of NF-κB in NaBT-mediated PTEN regulation, an adenovirus expressing the superrepressor of IκB-α (IκB-AA) (12) was used (12). HT29 cells were infected with either the adenovirus encoding hemagglutinin-tagged IκB-AA or the adenoviral control vector encoding green fluorescent protein (GFP). Infection was carried out for 1 h followed by the replacement of fresh medium. After incubation for 24 h, cells were treated with NaBT for an additional 24 h. As expected, IκB-α overexpression alone increased PTEN expression and enhanced NaBT-mediated PTEN induction (Fig. 3C, top); IκB-AA expression was confirmed by Western blot (Fig. 3C, bottom). The inhibition of NF-κB activation was confirmed by EMSA (data not shown). Conversely, we analyzed the effect of the expression of the p65 subunit of NF-κB after infection with the recombinant adenovirus Ad5p65. We found that overexpression of p65 protein decreased PTEN expression and attenuated NaBT-induced PTEN expression (Fig. 3D, top); p65 overexpression was confirmed and β-actin expression was constant, indicating equal protein loading (Fig. 3D, middle and bottom, respectively). Consistently, NaBT-mediated NF-κB inhibition was reversed by p65 overexpression as shown in Fig. 3E. These results provide evidence for the involvement of NF-κB in the regulation of NaBT-mediated PTEN induction.

Cross-talk between JNK and NF-κB in HT29 cells. Next, we assessed whether NF-κB affects JNK activity in HT29 cells. HT29 cells were infected with adenovirus encoding IκB-AA (Fig. 4A), adenovirus encoding p65 (Fig. 4B), or the adenoviral control vector...
encoding GFP. Activity of JNK1 or JNK2 was examined using GST-c-Jun protein as the substrate. NF-κB inhibition by overexpression of IκB-α increased JNK1 and JNK2 activity in HT29 cells, as shown in Fig. 4A. Consistent with these results, NF-κB activation by overexpression of p65 in HT29 cells decreased basal JNK1 activity and attenuated NaBT-mediated JNK activation (Fig. 4B). JNK1 and JNK2 expression levels were not altered with the transfections as noted in the Western blot (Fig. 4B, bottom).

Because JNK inhibition results in NF-κB activation in multiple myeloma cell lines (18), we next determined the effect of JNK inhibition on NF-κB activation by transfection of the NF-κB-luciferase plasmid, which contains four tandem copies of the NF-κB consensus sequence. Twenty-four hours after transfection, HT29 cells were pretreated with or without SP600125 (10 μmol/L) for 30 min followed by combination treatment with NaBT (5 mmol/L) for an additional 24 h. Treatment with NaBT decreased NF-κB transactivation compared with control (Fig. 4C). Moreover, treatment with the JNK inhibitor SP600125 significantly increased NF-κB reporter activity but only partially attenuated the NaBT-mediated NF-κB repression. NF-κB activation by JNK inhibition was further confirmed by the increased NF-κB reporter activity in cells transfected with JNK1 and JNK2 siRNA as compared with control siRNA transfection (Fig. 4D). Together, these results identify a negative regulation between JNK and NF-κB signaling, which plays an important role in the regulation of basal PTEN expression in HT29 cells.

Regulation of p27kip1 expression through the JNK/PTEN pathway in NaBT-treated HT29 cells. PTEN increases p27kip1 expression in various cells (4, 5, 36). We have shown that NaBT increases p27kip1 expression in intestinal cells (37). We next determined whether elevated PTEN expression contributed to the increase of p27kip1 expression. First, we assessed the expression of p27kip1 in HT29 cells overexpressing PTEN. Cells were infected with adenovirus vectors encoding β-gal (AdCA-LacZ; control) or PTEN (AdCA-PTEN). Twenty-four hours after infection, cells were lysed and p27kip1 expression was analyzed by Western blotting. As shown in Fig. 5A, overexpression of PTEN increased p27kip1 expression. To further confirm the role of PTEN in p27kip1 regulation, HT29 cells were transfected with nontargeting control siRNA or siRNA directed to PTEN. Twenty-four hours after transfection, cells were treated with NaBT for an additional 24 h, and p27kip1 expression was determined by Western blotting (Fig. 5B). Treatment with NaBT increased PTEN and p27kip1 expression. In agreement with the induction of p27kip1 by overexpression of PTEN, treatment with PTEN siRNA completely blocked PTEN expression and attenuated p27kip1 induction by NaBT, suggesting a role for PTEN in p27kip1 regulation in intestinal cells. Knockdown of PTEN did not completely block NaBT-induced p27kip1 expression, suggesting that a PTEN-independent pathway(s) is involved in NaBT-induced p27kip1 expression. In preliminary findings, we have shown that NaBT treatment increases expression of the forkhead transcription factor FOXO1; knockdown of FOXO1 attenuated NaBT-induced p27kip1 expression, suggesting that NaBT regulation of p27kip1 is through PTEN as well as FOXO1.3

We have shown that NF-κB activation decreased PTEN expression (12). To determine the effect of NF-κB activation on p27kip1 expression, HT29 cells were infected with adenovirus vectors encoding GFP (Ad5GFP; control) or p65 (Ad5p65). Twenty-four hours after infection, cells were treated with NaBT for an additional 24 h, and p27kip1 expression was analyzed by Western blot. In agreement with the inhibition of PTEN expression, overexpression of p65 decreased basal expression of p27kip1 and attenuated NaBT-induced p27kip1 expression (Fig. 5C).

Given that inhibition of JNK attenuated PTEN expression, we next determined the effect of JNK knockdown on p27kip1 expression. HT29 cells were transfected with nontargeting control siRNA or siRNA specifically directed to JNK1 or JNK2. Twenty-four hours after transfection, cells were treated with NaBT for an additional 24 h, and JNK1, JNK2, and p27kip1 expression was assayed by Western blot (Fig. 5D). Treatment with NaBT increased

p27\textsuperscript{kip1} expression. Transfection with either JNK1 or JNK2 siRNA attenuated NaBT-mediated p27\textsuperscript{kip1} induction; the knockdown of JNK1 or JNK2 was confirmed after treatment with JNK1 or JNK2 siRNA, respectively. Taken together, our findings show that NaBT-induced PTEN through JNK activation and NF-\(\kappa\)B inhibition, which was associated with the induction of p27\textsuperscript{kip1}.

**PTEN function in NaBT-induced differentiation and apoptosis.** Because PTEN is a potential tumor suppressor for colorectal cancer (1) and inhibition of PI3K enhances NaBT-mediated intestinal cell differentiation (6), we analyzed the role of PTEN in NaBT-induced differentiation in HT29 cells. Treatment with NaBT increased intestinal alkaline phosphatase activity (Fig. 6A), a differentiation marker for intestinal cells (6), and this increase was attenuated by transfection of siRNA directed to PTEN, suggesting a role for PTEN in NaBT-mediated differentiation.

Previously, we have found that inhibition of PI3K enhances NaBT-mediated apoptosis in human colon cancer cells (29). Next, we accessed the effect of PTEN knockdown on NaBT-induced HT29 cell death. NaBT induced obvious cell death as shown by increased DNA fragmentation (Fig. 6B), and this increase was attenuated by knockdown of PTEN using PTEN siRNA transfection. Collectively, our results suggest that PTEN plays a role in NaBT-mediated intestinal cell death and differentiation.

**Discussion**

In previous studies, we showed enhanced intestinal cell differentiation by PTEN (6) and defined a novel feedback regulation of PTEN by NF-\(\kappa\)B activation (12). In the present study, we further delineated the signaling pathways involved in this regulation. We show that NaBT, which induces intestinal cell differentiation, increased PTEN expression. This NaBT-mediated PTEN induction was a consequence of NF-\(\kappa\)B inhibition and JNK activation. Finally, we show that NaBT increased p27\textsuperscript{kip1} expression, a downstream target of PTEN by regulation of NF-\(\kappa\)B and JNK. Importantly, our findings identify regulation of PTEN by JNK and NF-\(\kappa\)B in intestinal cells.

Recently, we have shown that treatment with NaBT induced intestinal cell differentiation, which was associated with the activation of JNK (20). Conversely, inhibition of JNK attenuated NaBT-induced intestinal cell differentiation (21). JNK activity has been associated with intestinal cell death. For instance, TNF\(\alpha\)-induced apoptosis in IEC-6 intestinal cells was accompanied by the activation of JNK, and inhibition of JNK protected against TNF\(\alpha\)-induced apoptosis (38). Inhibition of JNK attenuated hydrogen peroxide–induced cell death in HT29 cells (39). Consistently, our previous results showed that overexpression of PTEN enhanced intestinal cell differentiation (6). Overexpression of PTEN in colorectal cancer cells resulted in cell cycle arrest and enhanced cell death through inhibition of PI3K (40). Our observation that the blockade of the JNK pathway by pharmacologic (i.e., SP600125) or genetic (i.e., transfection with JNK siRNA) mechanisms attenuated NaBT induction of PTEN strongly argues for a functional role for JNK activation in PTEN induction in intestinal cells.

Although in some cells (e.g., leukemia histone deacetylase inhibitor exposure has been associated with NF-\(\kappa\)B activation (41), several reports have shown inactivation of NF-\(\kappa\)B by NaBT in intestinal epithelial cells (42, 43). In agreement with these reports, we also showed an inhibitory role of NaBT in NF-\(\kappa\)B activity in HT29 cells. Although NaBT has been shown to decrease p50 in nuclear but not in cytosolic fractions of HT29 cells 12 h after NaBT treatment (44), our results showed that NaBT reduced p50 in both nuclear and cytosolic fractions with NaBT treatment for 48 h. NaBT inhibits TNF\(\alpha\)-induced NF-\(\kappa\)B activation, in part, by preventing the complete degradation of I\(\kappa\)B\(\alpha\) by reducing proteasome activity (42); however, our results showed that NaBT alone did not affect I\(\kappa\)B\(\alpha\) expression in HT29 cells. Because NF-\(\kappa\)B inhibition resulted in the induction of PTEN expression, our results strongly suggest the involvement of NF-\(\kappa\)B inhibition in NaBT-mediated PTEN induction.

Several studies have reported a cross-talk between the NF-\(\kappa\)B and JNK pathways (45, 46). The NF-\(\kappa\)B-mediated inhibition of JNK signaling is crucial for numerous physiologic processes, such as the response of the liver to injury and the survival of cells during an inflammatory reaction, as well as for chronic inflammatory diseases and cancers (16). In contrast, it has been reported that inhibition of NF-\(\kappa\)B leads to JNK activation and potentiates the lethality of certain apoptotic stimuli (e.g., TNF\(\alpha\); ref. 47). The importance of this antagonistic cross-talk between NF-\(\kappa\)B and JNK has also been documented recently in animal
models (44). In agreement with these findings, we showed that inhibition of NF-κB or BAX resulted in JNK activation. Activation of NF-κB decreased JNK activity, whereas inhibition of JNK increased NF-κB activity. Therefore, our results reveal a possible cross-talk mechanism by NF-κB and JNK on PTEN regulation in intestinal cells. Nevertheless, this mechanism may not contribute completely to NaBT-induced PTEN expression because our results show that JNK inhibition does not reverse NaBT-mediated NF-κB inhibition. Furthermore, NaBT increased JNK activity and, at the same time, did not affect NF-κB binding activity from 2 to 8 h. These results suggest that NF-κB and JNK are differentially involved in the regulation of NaBT-mediated PTEN expression. Our results show that JNK is involved at early time points and, together with NF-κB, at later stages in the regulation of NaBT-mediated expression.

NaBT induces intestinal cell differentiation associated with the induction of p27kip1 expression (37). The function of p27kip1 is required for intestinal cell differentiation (48). Overexpression of PTEN or inhibition of PI3K increased p27kip1 expression in various cells (4, 5). Considering that intestinal cell differentiation was enhanced by PTEN overexpression, we determined whether JNK activation increased p27kip1 and, if so, whether this effect was through JNK-dependent PTEN expression. We found that overexpression of PTEN or treatment with NaBT increased p27kip1 expression in HT29 cells; this induction was attenuated by transfection with PTEN siRNA, activation of NF-κB, or inhibition of JNK by transfection with JNK1 or JNK2 siRNA. Our findings show that NaBT increased p27kip1 expression through PTEN induction, which was mediated by JNK activation and NF-κB inhibition.

Previously, we showed that inhibition of PI3K or overexpression of PTEN increased TNF-related apoptosis inducing ligand (TRAIL) expression in intestinal cells (22); TRAIL treatment results in increased intestinal cell differentiation and p27kip1 expression (49). Recently, Yanase et al. (50) showed that activation of JNK leads to TRAIL induction in Daudi B lymphoma cells. Our future studies will determine the regulation of TRAIL expression by the JNK/PTEN pathway and the possible relationship to intestinal cell differentiation.

In summary, our current study provides important insights about the signaling mechanisms regulating PTEN expression and function in intestinal cells. Our results identify PTEN as a downstream target of the JNK and NF-κB pathway. Given the importance of p27kip1 in intestinal cell differentiation, JNK/PTEN and NF-κB/PTEN pathways may alter intestinal cell differentiation through the regulation of p27kip1 expression. Collectively, our results have important implications for normal intestinal homeostasis and colon carcinogenesis.

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