Involvement of Sp1 and Sp3 in differential regulation of human NHE3 promoter activity by sodium butyrate and IFN-γ/TNF-α

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Amin MR, Dudeja PK, Ramaswamy K, Malakooti J. Involvement of Sp1 and Sp3 in differential regulation of human NHE3 promoter activity by sodium butyrate and IFN-γ/TNF-α. Am J Physiol Gastrointest Liver Physiol 293: G374–G382, 2007. First published May 31, 2007; doi:10.1152/ajpgi.00128.2007.—Previously, we reported that IFN-γ and TNF-α downregulate the expression of the human Na+/H+ exchanger (NHE3) gene by modulating Sp1/Sp3 transcription factors in C2BBe1 cells. It is reported that butyrate inhibits IFN-γ and TNF-α signaling pathways. In this study, we have investigated the effect of sodium butyrate (NaB) and IFN-γ/TNF-α on human NHE3 promoter activity. In transient transfection studies, NaB (5 mM) led to 10-fold stimulation of NHE3 promoter activity after incubation for 24 h. With 5′-deletion analysis, the NaB-responsive region was mapped to the NHE3 core promoter, bp −95 to +5, which we had shown previously to confer responsiveness to IFN-γ/TNF-α. The stimulatory effect of NaB on the NHE3 promoter was reduced by 60% in the presence of IFN-γ/TNF-α. Mutually, the repressive effect of these cytokines was attenuated by NaB. Knockdown of Sp1 and Sp3 expression with small interfering RNA (siRNA) resulted in a significant resistance to NaB effects. NaB treatment showed no effect on Sp1 and Sp3 protein expression as assessed by Western blot analyses. Gel mobility shift assays with nuclear proteins from NaB-treated cells showed enhanced binding of Sp1 and Sp3 to the NHE3 promoter. The phosphatase inhibitor okadaic acid (200 nM) blocked the stimulatory effect of NaB on the NHE3 promoter. NaB effects on promoter expression were significantly attenuated by protein phosphatase (PP1)α and PP2Aα-specific siRNA transfection. Our data suggest that the differential regulation of NHE3 gene expression by NaB and IFN-γ/TNF-α is mediated through alternative pathways that converge on Sp1/Sp3.

sodium/hydrogen exchanger; C2BBe1 cell line

to date, nine members of the Na+/H+ exchanger (NHE) gene family have been identified and characterized in eukaryotic cells. The NHE gene family is involved in regulation of intracellular pH, cell volume, and neutral sodium absorption in the human intestine. NHE1, NHE2, and NHE3 are expressed in the human gastrointestinal tract. The protein products of the NHE gene family show characteristic subcellular localization. In polarized cells, NHE1 is localized to the basolateral membrane, NHE2, NHE3, and NHE8 are localized to the apical membrane, and NHE6, NHE7, and NHE9 are located intracellularly (4, 20, 46, 47). The importance of the NHE3 isoform in electrolyte and fluid homeostasis has been demonstrated in NHE3 knockout studies (45) as well as inhibition of its activity in diarrheal diseases (47).

There is increased expression of proinflammatory cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-1, IL-4, IL-6, IL-12, and IL-23 (15) in inflammatory bowel diseases (IBD). The elevated levels of these cytokines in the gut are involved in the pathogenesis of chronic intestinal inflammation causing malabsorption and diarrhea due to the inhibition of NHE2- and NHE3-mediated sodium transport and intestinal barrier dysfunction (11, 15, 35). IFN-γ and TNF-α have been shown to repress NHE3 gene expression (1, 35).

Butyrate decreases the expression of proinflammatory cytokines in IBD (23, 40); exerts immunomodulatory effects, such as downregulation of T cell responses, induction of Th1 cell anergy, and modulation of antigen presentation-associated molecules (17); and stimulates anti-inflammatory type 2 cytokines (e.g., IL-10) and downregulates type 1 cytokines (e.g., INF-γ) (40). Inability to utilize butyrate as a fuel by colonoocytes has been implicated in the pathogenesis of antibiotic-associated diarrhea (10) and ulcerative colitis (43). Among the short-chain fatty acids (SCFAs), sodium butyrate (NaB) seems to exert the most significant effect on colonic biology (18, 37). NaB is produced by bacterial fermentation of dietary fiber and acts as a physiological regulator of homeostasis of colonic epithelial cells by regulating the balance among proliferation, differentiation, and apoptosis (19).

We previously reported (1) that IFN-γ and TNF-α may downregulate the expression of the human NHE3 gene by cAMP-dependent protein kinase (PKA)-mediated phosphorylation of Sp1 and Sp3 transcription factors in C2BBe1 cells. Sp1, also called specificity protein 1, is a member of the C2-H2 zinc finger family (14) and has been implicated in the regulation of the human and rat NHE3 genes (1, 22, 30). Sp1 is involved in the regulation of a wide variety of genes, including housekeeping genes (24), the early promoter of SV40 (6), a number of growth factor genes (9), genes involved in proliferation (27), and genes encoding the extracellular matrix proteins (41). The transcriptional activity mediated by Sp1 appears to be promoter specific and in response to specific extracellular signals. Promoter activity may be affected either through a change in the absolute quantity of Sp1 molecules in the cell or by posttranslational modification of the transcription factor (13). It has been reported that Sp1 protein is phosphorylated by a number of kinases such as casein kinase II, DNA-dependent protein kinase, PKA, and protein kinase C (3, 26, 34, 36). It is noteworthy that serine/threonine phosphatases counteract Sp1 phosphorylation and stimulate gene expression (13, 25). NaB

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modulates gene expression via activation of serine/threonine protein phosphatases (12) and Sp1/Sp3 transcription factors (32, 44). It has been shown that NaB increases intestinal sodium absorption by stimulating NHE3 gene (21, 31); however, there is no report as to which transcription factor(s) is involved in the regulation of NHE3 gene by NaB. In addition, the SCFA butyrate has been shown to inhibit the IFN-γ and TNF-α signaling pathways (2, 23); however, the effect of NaB on the repressive activity of IFN-γ and TNF-α on the NHE3 promoter is unknown. In the present study, we investigated whether NaB counteracts the inhibitory effects of IFN-γ and TNF-α on the NHE3 promoter and the mechanism of its action in C2BBe1 cells. Our data suggest that NaB may attenuate the repressive effect of IFN-γ and TNF-α on the NHE3 promoter via protein phosphatase (PP)1 and PP2A protein phosphatase-mediated dephosphorylation of Sp1 and Sp3 transcription factors in C2BBe1 cells.

**EXPERIMENTAL PROCEDURES**

**Materials.** Commercial products were purchased as described previously (1). Briefly, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and Lipofectamine 2000 were obtained from Invitrogen life Technologies (Carlsbad, CA). IFN-γ, n-butryic acid (sodium salt), and transferrin were purchased from Sigma-Aldrich (St. Louis, MO). The gel shift assay core system and recombinant human TNF-α were from Promega (Madison, WI). The small interfering RNAs (siRNAs) against Sp1, Sp3, PP1α, and PP2Aα, control siRNA, and polyclonal anti-rabbit Sp1 and Sp3 and mouse monoclonal actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Okadaic acid was obtained from Calbiochem (La Jolla, CA).

**Promoter reporter plasmids.** Plasmids used for functional analysis of the NHE3 promoter activity were generated with pGL2-basic (Promega), which contains a promoterless luciferase reporter gene, as previously described (29).

**Cell culture and transfection.** C2BBe1 cells, obtained from the American Type Culture Collection (Manassas, VA), were maintained in culture as previously described (28). This cell line is a subclone derived from a heterogeneous population of Caco-2 cells and has been shown to undergo spontaneous differentiation as determined by the appearance of the characteristics of micovilli and the presence of the markers of differentiation (33). Cells were used between passages 3 and 20 and seeded at 5 × 10^4 cells/cm^2 onto collagen-coated 100-mm plastic plates in 10 ml of medium [DMEM containing 10% FBS, transferrin (10 μg/ml), 50 U/ml of penicillin G sodium, and 50 μg/ml streptomycin sulfate]. Before treatment with NaB or IFN-γ (30 ng/ml) and TNF-α (20 ng/ml) for various time periods, C2BBe1 cells were incubated in serum-reduced medium (0.5% FBS) for 24 h. For transient transfection studies, cells (1.8 × 10^6) were seeded onto collagen-coated 12-well plastic plates and cotransfected on the next day (~90% confluent) with one of the NHE3-luc reporter constructs and a plasmid expressing alkaline phosphatase for normalization to 100% FBS-supplemented DMEM. After 24 h of plasmid transfection the cells were treated with NaB for 24 h, and cells were harvested at 48 or 72 h after siRNA transfection. Reporter gene activities were determined according to the procedure described above. Cells transfected with specific siRNA for 48 h were used for NHE3 mRNA expression studies.

**RNA extraction and RT-polymerase chain reaction analysis.** Total RNA was isolated with RNA STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturer’s instructions. For cDNA synthesis, 5 μg of total RNA was reverse transcribed in the presence of oligo(dT), RNase Out, dNTPs, first-strand buffer, and Superscript II reverse transcriptase according to the Invitrogen protocol. Semiquantitative polymerase chain reactions (PCR) were performed with a Perkin-Elmer DNA thermal cycler and a Platinum PCR SuperMix kit (Invitrogen). The following primers were used for PCR amplifications: NHE3: sense 5′-CGACAGCTGGTCTGAAACC-3′ and antisense 5′-CTCACGCACTGATGCGGCT-3′; GAPDH: sense 5′-ATGGCACCGTCAAGGCTGAGA-3′ and antisense 5′-GAGTGCTGTGATCAG-3′. PCR conditions for GAPDH were denaturation at 95°C for 2 min, then the first 4 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, followed by 31 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s. PCR conditions for GAPDH were 20 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s. Final extension was at 72°C for 5 min. cDNA were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The amplification reactions yielded the expected DNA fragments: 484 bp (NHE3) and 371 bp (GAPDH).

**Western blot analysis.** Nuclear proteins (NP) were used for Sp1 and Sp3 Western immunoblotting. Proliferating C2BBe1 (~95% confluent) cells were serum starved in DMEM containing 0.5% FBS for 24 h and treated with NaB for various time periods as indicated. For Sp1 and Sp3, 20 μg of NP was separated by 10% SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membrane (Immobilon-P, Millipore). The Sp1 and Sp3 proteins were detected with anti-Sp1 and anti-Sp3 rabbit polyclonal antibodies, respectively. A peroxidase-linked goat anti-rabbit IgG was used as a secondary antibody. The complexes were detected with an enhanced chemiluminescence System (ECL plus, Amersham Pharmacia Bio- tech, Chicago, IL). The blots were reprobed for actin mouse monoclonal antibodies as loading control for Sp1 and Sp3, respectively. To evaluate reproducibility, these experiments were repeated at least three times.

**Gel mobility shift assay and supershift analysis.** All oligonucleotides for gel mobility shift assay (GMSA) were synthesized by Invitrogen Life Technologies. Complementary oligonucleotides were made double stranded by heating at 95°C for 5 min and slow cooling to 25°C in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The sequences of the sense strand of Sp1 probes were 5′-TGCGGG-GGCGGGGGGGGGCTC-3′, 5′-GGCGCGGGGGGGGGAGGGG-GGGG-3′, and 5′-CCCAGGGGGGGGGAGGGATCCGA-3′ located bp

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Mature RNA was obtained from C2BBe1 cells, serum-starved in DMEM containing 0.5% FBS for 24 h, to investigate the effect of NaB on NHE3 promoter activity, C2BBe1/H9253

Effect of NaB and IFN-γ on NHE3 promoter and identification of responsive region. Functional analysis of the 5’-deletion NHE3 promoter reporter constructs was carried out in C2BBe1 cells by transient transfection in the presence and absence of NaB and IFN-γ/TNF-α. Serum-starved cells were incubated with the cocktail of IFN-γ/TNF-α for 1 h before addition of NaB (5 mM) for 24 h. Among the 5’-deletion constructs, p−95/+5 showed maximum repression of 60% in response to IFN-γ/TNF-α, consistent with our previous results, and a maximum stimulation of 10-fold in response to NaB (Fig. 3A). The combined treatment with NaB and IFN-γ/TNF-α led to 60% reduction in reporter gene activity compared with NaB alone. These results indicate that response elements mediating the effects of NaB and IFN-γ/TNF-α are present in the bp −95 to +5 region of the NHE3 promoter. Similar reporter gene activities were obtained when C2BBe1 cells were first exposed to NaB for 1 h before treatment with IFN-γ/TNF-α and incubation continued for 24 h. The results obtained with p−1507/+131 are shown in Fig. 3B.

Role of Sp1/Sp3 in NaB-induced regulation of NHE3 promoter. We previously reported (1, 30) that the NHE3 promoter region from bp −95 to +5 was sufficient for basal expression of the NHE3 promoter and that transcription factors Sp1 and Sp3 are involved in this process. Since the NHE3 core promoter contains several Sp1/Sp3 binding sites (Fig. 3C) and NaB treatment showed maximum effects at this region, we hypothesized that NaB response may be mediated by the Sp1 and Sp3 transcription factors. Recently, we showed (1) that the repressive effect of IFN-γ/TNF-α on the NHE3 promoter is mediated through the same region by the Sp1/Sp3 transcription factors.

To investigate the involvement of Sp1 and Sp3 transcription factors in the NaB-mediated stimulation of NHE3 promoter activity, the effects of siRNAs specific for Sp1 and/or Sp3 were determined. C2BBe1 cells were transfected with Sp1 and Sp3 siRNAs individually or simultaneously and then transfected

-89 to −67; bp −64 to −42, and bp −28 to −7, respectively, of the NHE3 promoter. The probes were end-labeled with T4-polynucleotide kinase and [γ-32P]ATP (Amersham, Arlington Heights, IL). Binding reactions were initiated by incubating 5 µl of NP and 2 µl of binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 0.25 µg/µl poly(dI-dC), 20% glycerol (Promega)) for 10 min at room temperature in a volume of 10 µl; ~40,000 cpm of labeled probes were then added and incubated for 25 min at room temperature. In competition assays, the unlabeled probes were added to the reaction mixture for 10 min before the labeled probe was added. For supershift analysis, anti-Sp1 and anti-Sp3 rabbit polyclonal antibodies were incubated with the binding reaction mixtures for 30 min at room temperature before electrophoresis on 5% polyacrylamide gel in 0.25× Tris-borate-EDTA running buffer. Gels were dried and visualized by autoradiography.

Statistical analyses. Differences between two groups were evaluated with Student’s t-test. P < 0.05 was used to indicate statistical significance.

RESULTS

Dose-dependent effects of NaB on NHE3 core promoter construct p−95/+5 in C2BBe1 cells. To assess the dose-response effect of NaB on NHE3 promoter activity, C2BBe1 cells were transiently transfected with the NHE3 core promoter, serum-starved in DMEM containing 0.5% FBS for 24 h, and incubated with different doses of NaB for 24 h. NaB at a 5 mM concentration led to maximum (~10-fold) stimulation of NHE3 promoter activity after incubation for 24 h (Fig. 1).

Effects of NaB and IFN-γ/TNF-α on expression of NHE3 mRNA in C2BBe1 cells. We used RT-PCR experiments to investigate the effect of NaB and IFN-γ/TNF-α on NHE3 mRNA expression. Total RNA was obtained from C2BBe1 cells treated with NaB, IFN-γ/TNF-α, and a combination of both NaB and IFN-γ/TNF-α. Subsequently, reverse transcription and PCR amplifications were performed with NHE3 and GAPDH gene-specific primers. As shown in Fig. 2, the stimulatory effect of NaB on NHE3 mRNA expression is reduced by ~50% in the presence of IFN-γ/TNF-α.

Effects of NaB and IFN-γ/TNF-α on NHE3 promoter activity, the effects of siRNAs specific for Sp1 and Sp3 were determined. C2BBe1 cells were transfected with Sp1 and Sp3 siRNAs individually or simultaneously and then transfected

![Fig. 1. The dose-dependent effects of sodium butyrate (NaB) on Na+/H+ exchanger (NHE3) core promoter construct p−95/+5. Transiently transfected C2BBe1 cells were serum starved in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.5% fetal bovine serum (FBS) for 24 h and then incubated with different doses of NaB for 24 h. Forty-eight hours after transfection, cells were harvested. Luciferase activities of both treated and untreated samples are presented relative to the normalized activity of the promoterless pGL2-basic construct. Values are means ± SE for triplicate assays from 3 different experiments performed on different days. Statistical analyses were done by Student’s t-test. *P < 0.05, significant difference between control and NaB-treated cells (n = 4).](image-url)

![Fig. 2. Effects of NaB and interferon (IFN)-γ/Tumor necrosis factor (TNF)-α on the expression of NHE3 mRNA in C2BBe1 cells. Proliferating cells were serum starved in DMEM containing 0.5% FBS for 24 h and treated with 5 mM NaB and IFN-γ/TNF-α for 6 h, and total RNA was prepared as described in EXPERIMENTAL PROCEDURES. Total RNA was subjected to reverse transcription and subsequent PCR amplification using NHE3 and GAPDH gene-specific primers. M, molecular weight standard; C, control; I, IFN-γ (30 ng/ml); T, TNF-α (20 ng/ml).](image-url)
again with p=95+/+/5 and treated with NaB as described in experimental procedures. The results of these studies revealed that under these experimental conditions the NHE3 core promoter showed much less responsiveness to NaB treatments compared with the control. We previously showed (1) that silencing of Sp1 and Sp3 caused decreased production of Sp1 and Sp3 proteins and led to decreased NHE3 mRNA expression. The sensitivity of NHE3 core promoter activity to NaB decreased to 50% after transfection with Sp1 (Fig. 4A) or Sp3 (Fig. 4B) siRNAs. NaB lost 80% of its stimulatory effect on NHE3 core promoter activity after simultaneous transfection with Sp1 and Sp3 siRNAs (Fig. 4C). These results indicate that stimulation of the NHE3 promoter activity in response to NaB is mediated via Sp1/Sp3 transcription factors.

Time course effects of NaB on expression of Sp1 and Sp3 proteins in C2BBe1 cells. To examine the effects of NaB on Sp1 and Sp3 protein expression, Western blot experiments were performed with nuclear proteins prepared from cells treated with NaB as indicated in Fig. 4 and blots were probed with anti-Sp1, -Sp3, and -actin antibodies. Sp1 (Fig. 5A) and Sp3 (Fig. 5B) protein levels remained unaffected at all time periods after C2BBe1 cells were treated with NaB. Actin expression was used as loading control for Sp1 and Sp3 blots. Previously, we showed (1) that IFN-γ/TNF-α reduced the DNA binding affinity of the Sp1 and Sp3 transcription factors without affecting their protein levels. NaB or IFN-γ/TNF-α treatment also showed no effect on the expression of Sp1 and Sp3 mRNA (data not shown).

Analysis of DNA binding activities of NP from NaB-treated cells with Sp1 sites of NHE3 promoter. Our previous studies have established that bp = 95 to +5 of the human NHE3 proximal promoter region, containing multiple Sp1 binding sites, is sufficient to promote basal promoter activity and that Sp1 and Sp3 proteins bind to this region at bp = 89 to 67. Figure 6A shows the results of a representative GMSA using the Sp1 probe (bp = 89 to 67) and NP from NaB-treated and untreated C2BBe1 cells. Three DNA-protein complexes were detected in both nuclear extracts, which showed a pattern similar to the Sp1 and Sp3 complexes identified previously (Fig. 6A, lanes 1 and 6). The binding specificity of these complexes was verified by competition assays with 100-fold molar excess of unlabeled Sp1 consensus sequence (Fig. 6A, lane 5). Unlabeled Sp1 oligonucleotide completely competed out Sp1/Sp3 DNA-protein complexes, suggesting that the protein components of these complexes were Sp1 related. The identities of the proteins present in these complexes were confirmed by supershift assays. Incubation of anti-Sp1 rabbit polyclonal antibody with NP-probe mixtures resulted in the formation of a slow-migrating supershift band (Fig. 6A, lanes 2, 4, 7, 10). Anti-Sp3 rabbit polyclonal antibody completely removed Sp3 protein-probe complex, indicating that one of the protein components of this complex was Sp3 (Fig. 6A, lanes 3, 4, 8, 10). These results confirm that the Sp1 binding site (bp = 89 to 67) of the human NHE3 gene promoter binds to Sp1 and Sp3 transcription factors, and NaB increases the binding activity of Sp1 and Sp3 proteins to the NHE3 promoter. The other two Sp1 sites at bp = 64 to 42 and 28 to 7 showed results similar to the Sp1 site at bp = 89 to 67 with NP from C2BBe1 cells treated with NaB (Fig. 6, B and C). An approximately threefold increase in the intensity of Sp1 and Sp3 DNA-protein complexes was observed in NP from NaB-treated...
C2BBe1 cells compared with untreated cells (Fig. 6A, lanes 1 and 6, B, and C). In our previous study (1), we observed an 50% reduction in the intensity of Sp1/Sp3 DNA-protein complexes in NP from IFN-γ/TNF-α treated C2BBe1 cells.

Effect of phosphatase inhibitor okadaic acid on NaB-induced regulation of NHE3 promoter. C2BBe1 cells were transiently transfected with the pNHE35 promoter reporter construct and then treated with NaB in the presence and absence of the phosphatase inhibitor okadaic acid (100 or 200 nM) for 24 h. Cells were preincubated with the inhibitor for 1 h. As a control, transfected cells were incubated with 0.5% FBS-supplemented DMEM. Okadaic acid at 100 nM partially and at 200 nM almost completely blocked the stimulatory effect of NaB on the NHE3 promoter in transient transfection assays (Fig. 7). These results suggest that okadaic acid blocks the stimulatory effect of NaB on the NHE3 promoter by inhibiting NaB-induced serine/threonine protein phosphatases in C2BBe1 cells.

Role of PP1 and PP2A protein phosphatases in regulation of NHE3 promoter. It has been reported that NaB treatment increases PP1 and PP2A in hepatoma tissue culture cells (15). The involvement of serine/threonine protein phosphatases PP1A and PP2A has been shown in the dephosphorylation of Sp1 transcription factor (13, 25). Therefore, we speculated that NaB-induced stimulation of NHE3 promoter activity might be via serine/threonine phosphatase-mediated dephosphorylation of Sp1/Sp3 transcription factors. To investigate the involvement of PP1α and PP2Aα protein phosphatases in the regulation of NHE3 promoter activity under basal and stimulated conditions, the effects of siRNAs specific for PP1α and/or PP2Aα were determined. Proliferating C2BBe1 cells were...
treated with PP1α and PP2Aα siRNAs individually or simultaneously and then transfected with p−95/+5 and treated with NaB as described in Experimental procedures. Simultaneous treatment of C2BBe1 cells with PP1α and PP2Aα siRNAs resulted in ~80% and ~65% inhibition of the promoter activity at 48 and 72 h, respectively (Fig. 8A). The results of these studies also revealed that ~70% stimulatory effect of NaB on the NHE3 core promoter was abolished by the simultaneous treatment of PP1α and PP2Aα siRNAs (Fig. 8B). Silencing of PP1α and PP2Aα phosphatases caused decreased expression of NHE3 gene (Fig. 8C). In addition, our previous studies (1) showed a decrease in Sp1-DNA interaction in NP from cells grown in the presence of different phosphatase inhibitors.

Discussion

We previously reported (1) that IFN-γ and TNF-α down-regulate the expression of the human NHE3 gene, possibly through a mechanism involving the phosphorylation of the Sp1 and Sp3 transcription factors, in C2BBe1 cells. We have determined that the IFN-γ/TNF-α response elements are located in the bp −95 to +5 region of the NHE3 promoter, and plasmid p−95/+5 showed ~60% repression of promoter activity in response to IFN-γ/TNF-α treatment (1). It has been shown that the SCFA butyrate inhibits the stimulatory effects of IFN-γ and TNF-α on expression of various genes (2, 23). In the present study, we investigated whether NaB can counteract the inhibitory effects of IFN-γ/TNF-α on the NHE3 promoter and the mechanism of its action in C2BBe1 cells. Our present data indicate that NaB enhances the NHE3 promoter activity, and the maximum stimulatory effect of NaB on the NHE3 promoter (~10-fold) is seen at the core promoter level, which also harbors the IFN-γ/TNF-α-responsive element. The stimulatory effect of NaB on endogenous NHE3 mRNA expression is reduced by 50% in the

Fig. 6. Analysis of DNA binding activity of the nuclear proteins from NaB-treated C2BBe1 cells and Sp1 binding sites of the NHE3 promoter. Gel mobility shift assays (GMSAs) were performed with Sp1 probes of the NHE3 promoter and nuclear proteins from proliferating C2BBe1 cells treated with 5 mM NaB for 6 h. Binding reactions were performed as described in Experimental procedures. Three oligonucleotides harboring Sp1 binding sites at −89 to −67 (A), −64 to −42 (B), and −28 to −7 (C) were used as end-labeled probes in GMSAs. A: lanes 1 and 6 show Sp1/Sp3 DNA-protein complexes between nuclear proteins from untreated and 5 mM NaB-treated C2BBe1 cells, respectively. The binding specificity of these complexes was examined by competition assays in which excess unlabeled Sp1-specific probe (lanes 2, 3, 4, 7, 10). Anti-Sp3 rabbit polyclonal antibody completely removed Sp3 protein-probe complex (lanes 3, 4, 8, 10). Anti-Egr-1 rabbit polyclonal antibody did not affect Sp1/Sp3 binding with the NHE3 promoter (lane 9). U and T, nuclear proteins from untreated or NaB-treated C2BBe1 cells, respectively. Results shown are representative of 3 separate similar experiments.

Fig. 7. Role of the phosphatase inhibitor okadaic acid (OA) in NaB-induced regulation of the NHE3 promoter. C2BBe1 cells were transiently transfected with the p−95/+5 NHE3 promoter reporter constructs and serum starved in DMEM containing 0.5% FBS for 24 h. Cells were incubated with OA for 1 h before addition of NaB and then incubated with 5 mM NaB, and incubation continued for 24 h. Results shown are representative of at least 3 separate experiments. *P = 0.0001 and **P = 0.002, statistical difference from control values determined by Student’s t-test (n = 4).
presence of IFN-γ/TNF-α. Since the NHE3 core promoter contains several Sp1/Sp3 binding sites and both NaB and IFN-γ/TNF-α showed their maximum effects on the NHE3 promoter at the core promoter region, we hypothesized that NaB might attenuate the repressive effect of IFN-γ/TNF-α on NHE3 promoter activity by modulating the Sp1 family transcription factors.

To examine the involvement of the Sp1 and Sp3 transcription factors in mediating the effect of NaB on the NHE3 promoter in C2BBe1 cells, we have used siRNAs specific for Sp1 and Sp3 to knock down the expression of these factors. We show that the knockdown of both Sp1 and Sp3 by specific siRNA, individually or simultaneously, leads to a significant reduction in the stimulatory effect of NaB on NHE3 promoter activity. NaB-induced NHE3 promoter activity was decreased by ~80% by simultaneous treatment with Sp1 and Sp3 siRNAs. Thus our overall results from transient transfection, siRNA, and DNA-binding assays indicate that NaB mediates its stimulatory effect on the NHE3 promoter by modulating Sp1/Sp3 transcription factors. In addition, we showed by RT-PCR and Western blot analyses that NaB treatment had no effect on the Sp1 and Sp3 mRNA (data not shown) and protein expression in C2BBe1 cells. However, GMSA using a probe spanning the Sp1 binding site from bp −89 to −67 and the nuclear proteins from C2BBe1 cells treated with NaB showed approximately threefold increase in the intensity of both Sp1/Sp3 DNA-protein complexes compared with NP from untreated cells. These results suggest that the NaB-induced increased binding activity of these transcription factors to the NHE3 promoter may be mediated by posttranslational modification of Sp1/Sp3 transcription factors.

Sp family transcription factors are subject to different forms of posttranslational modifications, including phosphorylation and dephosphorylation. We found that ~80% of the stimulatory effect of NaB on the NHE3 promoter was blocked by okadaic acid treatment (200 nM) in transiently transfected cells and that ~70% stimulatory effect of NaB on the NHE3 core promoter was abolished by combined cotransfection of PP1α and PP2Aα siRNAs in C2BBe1 cells. These data suggest that NaB stimulates the NHE3 gene by activating a phosphatase-signaling pathway. Cuisset et al. (12) have shown that serine/threonine protein phosphatases could act as molecular intermediates in the signaling pathway used by NaB to modulate histone H1 and c-myc gene expression. Okadaic acid is known to be a potent inhibitor of the serine/threonine protein phosphatase family, including PP1A and PP2A (38, 42). Okadaic acid binds to the catalytic subunit of phosphatases and inhibits their enzymatic activity (5). Kinases and phosphatases maintain the phosphorylation/dephosphorylation state of the cellular proteins. When phosphatase activity is inhibited, the activity of cellular kinases appears indirectly to become upregulated. In this regard, we showed previously (1) that the DNA binding affinity of Sp1 and Sp3 transcription factors to the NHE3 promoter is reduced subsequent to treatment with various phosphatase inhibitors. These results support the notion that okadaic acid blocks the activity of a cellular phosphatase and mediates its stimulatory effect on the NHE3 promoter by modulating Sp1/Sp3 transcription factors.
leads to increased phosphorylation of Sp1/Sp3 transcription factors, and its decreased binding affinity for the NHE3 promoter.

It has been reported that NaB increases intestinal sodium absorption by stimulating NHE3 gene expression (21, 31), but there is no clear evidence for how NaB regulates the human NHE3 promoter activity. Kiel et al. (21) have shown that butyrate is involved in regulating rat NHE3 promoter activity; however, the factors mediating its effect were not studied. Furthermore, they reported that NaB effects on the rat NHE3 promoter depended on the activity of serine/threonine kinases, in particular PKA. They also indicated that inhibition of PP1 promoter depended on the activity of serine/threonine kinases. Furthermore, they reported that NaB effects on the rat NHE3 absorption by stimulating NHE3 gene expression (21, 31), but it was not clear if the fold increase in NHE3 promoter activity after NaB treatment. Our studies showed that okadaic acid (200 nM) almost completely abolished the NaB-mediated human NHE3 promoter stimulation (Fig. 7), and at a high dose (1 μM) it was toxic to the C2BBe1 cells (data not shown). This discrepancy may be attributed to the intrinsic differences between the human and rat NHE3 promoters, 2) the use of a high concentration of okadaic acid (1 μM) in the study mentioned above, and 3) the fact that the incubation time with both okadaic acid and NaB was significantly lower in that study (6 h vs. 24 h).

Several mechanisms of action of butyrate have been proposed, including changes in the chromatin structure through inhibition of histone deacetylases (8, 44), dephosphorylation of retinoblastoma protein (7, 39), activation of protein phosphatases (12), and transcriptional regulation via Sp1/Sp3 binding sites (32, 44). The serine/threonine phosphatases have been shown to cause dephosphorylation of Sp1 and stimulate gene expression (13, 25). In the present study, we found that the stimulatory effect of NaB on the human NHE3 promoter was blocked by okadaic acid treatment in transient transfection studies. Furthermore, NHE3 promoter activation by NaB was significantly attenuated (~70%) by reduction of PP1α and PP2Aα phosphatases by siRNAs. NaB treatment caused increased binding of Sp1/Sp3 transcription factors to the NHE3 promoter, without affecting the protein levels of Sp1 and Sp3. These results suggest that NaB-induced NHE3 promoter activity may be mediated via a phosphatase-induced dephosphorylation of Sp1 and Sp3 transcription factors.

The SCFA butyrate inhibits IFN-γ signaling by modulating STAT1 transcription factor (23, 40); whether STAT1 plays a role in the NHE3 promoter activity in response to these cytokines remains to be determined. The results of the present study suggest that NaB may inhibit IFN-γ/TNF-α-mediated signaling pathway on the NHE3 promoter by modulating the phosphorylation status of Sp1/Sp3 proteins in C2BBe1 cells. In addition, we have shown that NaB-induced upregulation of the NHE3 promoter may be mediated by PP1 and PP2A protein phosphatase-induced dephosphorylation of Sp1 and Sp3 transcription factors. Previously, we showed (1) that IFN-γ and TNF-α downregulate the expression of human NHE3 gene, possibly via PKA-mediated phosphorylation of Sp1/Sp3 transcription factors. In transient transfection studies, simultaneous treatment with NaB and IFN-γ/TNF-α led to a promoter activity that appears to be the outcome of the mutual effects of positive regulation by NaB and negative regulation by IFN-γ/TNF-α. Thus we conclude that an interplay between NaB-induced dephosphorylation and IFN-γ/TNF-α-induced phosphorylation of the Sp1/Sp3 proteins may determine NHE3 promoter expression in the presence of NaB and IFN-γ/TNF-α.

**REFERENCES**


10. Clausen MR, Bonnen H, Tvende M, Mortensen PB. Colonic fermenta-


