Na\(^+/\)H\(^+\) exchanger blockade inhibits enterocyte inflammatory response and protects against colitis

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THE INTESTINAL INFLAMMATION during inflammatory bowel disease (IBD) is a result of a complex interplay of immune and nonimmune cell interactions (12, 49). Emerging evidence indicates that of all the classical “nonimmune” cells present in the gut, the epithelial cells of the mucosa may play a central role in inducing and maintaining intestinal inflammation. During gut inflammation, intestinal epithelial cells (IECs) receive their activating signals from basically two sources: 1) the classical immune cells of the gut via humoral factors, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and 2) direct interactions with bacteria and bacterial products. These signals activate IECs to produce a wide range of inflammatory mediators, including the chemokine IL-8 (13, 55) and the free radical nitric oxide (28, 42, 54). Central to this inflammatory response is the transcription factor nuclear factor-κB (NF-κB), whose activation is responsible for the transcription of inflammatory genes in IECs during IBD (24). IL-8 is a powerful neutrophil chemoattractant and activator. Colonic IL-8 levels correlate with the macroscopic grade of local inflammation in IBD patients, in which large numbers of neutrophils are found in crypt abscesses (6, 33). In addition, IL-8 neutralization has been shown (19, 27) to abolish the neutrophil-activating effect of rectal dialysate or colonic organ cultures taken from IBD patients. Thus it appears that IL-8 of epithelial origin plays an important role in the amplification and/or maintenance of intestinal inflammation in IBD patients.

Na\(^+/\)H\(^+\) exchangers (antiporters, NHE) are a family of ubiquitous plasma membrane transport proteins that catalyze the exchange of extracellular Na\(^+\) for intracellular H\(^+\) (8, 15). Recent molecular cloning studies (50, 56, 57) have confirmed that NHEs constitute a gene family from which seven mammalian isoforms (NHE1, NHE2, NHE3, NHE4, NHE5, NHE6, and NHE7) have been cloned and sequenced. IECs have been shown (22, 31, 51) to express NHE1, NHE2, NHE3, and NHE4, but the exact function of the various isoforms is unknown. The “housekeeping” NHE1 is present on the basolateral membrane, where it is involved in intracellular pH maintenance and cell volume regulation. The apically expressed NHE3 plays an important role in Na\(^+\) bicarbonate, and water reabsorption (7). NHEs are reversibly and selectively inhibited by the diuretic drug amiloride and its analogs as well as by a variety of nonrelated drugs, including cimetidine, clonidine, and harmaline (50, 56).

Evidence indicates that NHEs are rapidly activated in response to a variety of inflammatory signals, such as IL-1 (5), TNF-α (52), interferon-γ (IFN-γ) (41) and lipopolysaccharide (LPS) (39, 52). Conversely, NHEs have been shown to regulate the inflammatory func-
tions of “professional” inflammatory cells, including monocytes (44), macrophages (35, 41), and neutrophils (45, 53). We hypothesized that NHEs may regulate the IEC inflammatory response because various extracellular stimuli present during gut inflammation, such as cytokines, bacteria, and bacterial products, activate NHEs and NHEs are involved in the regulation of inflammatory processes. Our results demonstrate an essential role for NHEs in mediating the IEC inflammatory response. We show that a functional NHE is required for both maximal NF-κB activation and IL-8 production in IECs. Furthermore, we demonstrate that the NHE regulation of intestinal inflammation is also operational in vivo, because NHE inhibition dramatically attenuates disease activity in the mouse dextran sulfate model of IBD.

MATERIALS AND METHODS

In Vitro Studies

Cell lines. The human colon cancer cell lines HT-29 and Caco-2 were obtained from American Type Culture Collection (Manassas, VA). HT-29 cells were grown in modified McCoy’s 5A medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Caco-2 cells were grown in DMEM with high glucose containing 10% fetal bovine serum (26).

Drugs and reagents. Amiloride HCl, 5-(N-methyl-N-isobutyl)amiloride (MIA), and 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) were obtained from Research Biochemicals (Natick, MA). Cimetidine, harmaline, and clonidine were purchased from Sigma (St. Louis, MO). All NHE inhibitors were dissolved in DMSO. Human IL-1β and TNF-α were obtained from R&D Systems (Minneapolis, MN). The p42/p44 pathway inhibitor PD-98059 was purchased from Calbiochem (San Diego, CA). LPS (Escherichia coli 055:B5) and pyrrolidinedithiocarbamate were purchased from Sigma. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Fisher Scientific (Pittsburgh, PA).

IL-8 measurement. To study the effect of NHE inhibitors on IL-8 production, cells in 96-well plates were treated with IL-1β (20 ng/ml), TNF-α (20 ng/ml), or LPS (10 μg/ml) for 24 h. However, because harmaline was toxic to the cells when the incubation lasted for 24 h, the effect of these agents on IL-8 production was tested 4 h after IL-1β stimulation. Furthermore, the effect of both MIA and EIPA was tested 4 h after stimulation with IL-1β. Amiloride or its vehicle (0.5% DMSO) was added to the cells at various time points before and after stimulation with cytokines or LPS. Human IL-8 levels were determined from the cell supernatants using commercially available ELISA kits (R&D Systems), according to the manufacturer’s instructions.

Western blot analysis. HT-29 cells in six-well plates were pretreated with amiloride (300 μM) or vehicle (0.5% DMSO), and 30 min later the cells were stimulated with IL-1β (20 ng/ml) for 15 min. After being washed with PBS, the cells were lysed by the addition of modified radioimmunoprecipitation buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25% Na deoxycholate, 1% Nonidet P-40, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM Na3VO4]. The lysates were transferred to Eppendorf tubes and centrifuged at 15,000 g, and the supernatant was recovered. Protein concentrations were determined using the Bio-Rad protein assay (Hercules, CA). Ten micrograms of sample were separated on a 8–16% Tris-glycine gel (Invitrogen) and transferred to a nitrocellulose membrane. The membranes were probed with anti-phospho-mitogen-activated protein kinase (MAPK) antibody (p42/p44; Promega, Madison, WI) and subsequently incubated with a secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (Boehringer-Mannheim, Indianapolis, IN). Bands were detected using enhanced chemiluminescence Western blotting detection reagent (Amersham Life Science, Arlington Heights, IL).

RNA isolation and RT-PCR. Total RNA was isolated from HT-29 and Caco-2 cells using TRIzol reagent (Invitrogen). RNA RT was performed using Moloney murine leukemia virus RT from Perkin-Elmer (50 U/μl; Foster City, CA). RNA (5 μg) was transcribed in a 20 μl reaction containing 10.7 μl of RNA, 2 μl of 10× PCR buffer, 2 μl of 10 mM dNTP mix, 2 μl of 25 mM MgCl2, 2 μl of 100 mM dithiothreitol (DTT), 0.5 μl of RNase inhibitor (Perkin-Elmer, 20 U/μl), 0.5 μl of 50 mM oligo(dT)16, and 0.3 μl of RT. The reaction was incubated at 42°C for 15 min for reverse transcription. Thereafter, the RT was activated at 99°C for 5 min. RT-generated DNA was amplified using Expand high-fidelity PCR system (Boehringer-Mannheim). The reaction buffer (25 μl) contained 2 μl of cDNA, water, 2.5 μl of PCR buffer, 1.5 μl of 25 mM MgCl2, 1 μl of 10 mM dNTP mix, 0.5 μl of 10 μM oligonucleotide primer (each), and 0.2 μl enzyme. cDNA was amplified using the following primers: IL-8 (36), 5′-ATGACTTCAACAGTGGCCGTGGCT-3′ (sense) and 5′-TCTCACGCTCTTCTAAAAAATCCT-3′ (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CGGATTTACACGGATTGTTGTTGAT-3′ (sense) and 5′-AGCCCTTCTCATGGTTGGTAAC-3′ (antisense). The conditions were as follows: an initial denaturation at 94°C for 5 min; 27 and 23 cycles at 94°C for 30 s for IL-8 and GAPDH, respectively; 58°C for 45 s; 72°C for 45 s; and 72°C for 7 min. The expected PCR products were 289 and 306 bp for IL-8 and GAPDH, respectively. The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

NF-κB electrophoretic mobility shift assay and supershift assay. IECs were stimulated with IL-1β (20 ng/ml; R&D Systems) for 45 min, and nuclear protein extracts were prepared as described previously (11, 34). To determine the effect of NHE blockade, we pretreated cells with amiloride (300 μM), harmaline (3 mM), cimetidine (6 mM), clonidine (3 mM), or vehicle (0.5% DMSO) 30 min before IL-1β stimulation. All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were washed twice with PBS and harvested by scraping into 1 ml of PBS and pelleted at 6,000 rpm for 5 min. The pellet was resuspended in 1 packed cell volume of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl2, 0.2% vol/vol Nonidet P-40, 1 mM DTT, and 0.1 mM PMSF) and incubated for 5 min with occasional vortexing. After centrifugation at 6,000 rpm, 1 cell pellet volume of extraction buffer (20 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl2, 25% vol/vol glycerol, 1 mM DTT and 0.5 mM PMSF) was added to the nuclear pellet and incubated on ice for 15 min with occasional vortexing. Nuclear proteins were isolated by centrifugation at 14,000 g for 15 min. Protein concentrations were determined using the Bio-Rad protein assay. Nuclear extracts were stored at −70°C until used for electrophoretic mobility shift assay (EMSA). The oligonucleotide probe used for the EMSA was purchased from Promega. Oligonucleotide probes were labeled with [γ-32P]ATP using T4 polynucle-
otide kinase (Invitrogen) and purified in Bio-Spin chromatography columns (Bio-Rad). For the EMSA analysis, 10 μg of nuclear proteins were preincubated with EMSA buffer [12 mM HEPES, pH 7.9, 4 mM Tris·HCl, pH 7.9, 25 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 50 ng/ml poly(dI-dC), 12% glycerol (vol/vol), and 0.2 mM PMSF] on ice for 10 min before the radiolabeled oligonucleotide was added for an additional 25 min. The specificities of the binding reactions were tested by incubating duplicate samples with 100-fold molar excess of the unlabeled oligonucleotide probe. Protein-nucleic acid complexes were resolved using a nondenaturing polyacrylamide gel consisting of 5% acrylamide (acrylamide/bisacrylamide, 29:1) and run in 0.5 M/H11003 TBE (45 mM Tris·HCl, 45 mM boric acid, and 1 mM EDTA) for 1 h at a constant current (30 mA). Gels were transferred to Whatman 3M paper, dried under transfection. Cells were transfected with 3 well of a 24-well tissue culture dish 1 day before transient added for an additional 25 min. The speci-

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Serum and tissue samples were harvested after 2 days of treatment, and the homogenates were cleared by centrifugation (10,000 g, 15 min) and stored at –70°C until analysis. From frozen samples, the protein concentration was determined by the bicinchoninic acid (BCA) assay (Pierce) and the absorbance at 562 nm was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as milligrams of protein per milligram of DNA.

**Measurement of mitochondrial respiration.** Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of MTT to formazan. Cells in 96-well plates were incubated with MTT (0.5 mg/ml) for 40 min at 37°C. The culture medium was removed by aspiration, and the cultures were solubilized in DMSO (100 μl). The extent of the reduction of MTT to formazan within cells was quantitated by an optical density measurement at 550 nm using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as milligrams of protein.
to ~60%. The possibility that the amiloride suppression of IL-8 production was due to a decrease in cell viability by this inhibitor was excluded using an MTT assay, which showed that amiloride did not suppress mitochondrial respiration in the 1–300 μM concentration range (Fig. 1B). The effect of higher concentrations of amiloride on IL-8 production was not investigated, because amiloride at 1,000 μM was toxic to the cells (data not shown).

To examine whether the effect of amiloride was cell-type specific, we investigated the effect of this agent on IL-1β-induced IL-8 production in the Caco-2 human epithelial cell line. Amiloride suppressed IL-8 production by these cells as well (Fig. 1C), with a similar potency to that observed in HT-29 cells. This observation suggests that the reduction of IL-8 production by NHE blockade is not cell-type specific, but rather a general phenomenon. To determine whether the inhibitory effect of amiloride treatment was time dependent, we administered 300 μM amiloride 30 min before, concurrent with, or 0.5, 1.5, 3, 4.5, 6, 7.5, or 9 h after stimulation with IL-1β. As shown in Fig. 2, concomitant treatment with amiloride resulted in maximal suppression of IL-8 production, which amounted to 57 ± 8.2% (n = 6, P < 0.01). In addition, a significant inhibition of IL-8 production was also observed up to 4.5 h after IL-1β stimulation. This finding demonstrates that the amiloride-induced suppression of IL-8 production does not require pretreatment.

In subsequent experiments, we examined the effect of selective inhibition of NHEs by the amiloride analogs MIA and EIPA on IL-1β-induced IL-8 production by HT-29 cells. Figure 3 demonstrates that both MIA and EIPA suppressed IL-8 production. MIA suppressed IL-8 production with an IC50 of ~10 nM (Fig. 3A). MIA was most efficacious at 100 nM; however, at higher concentrations, MIA became less effective. EIPA had a biphasic effect (Fig. 3B). The first phase of the EIPA suppression of IL-1β-stimulated IL-8 production reached its maximum at 300 nM, followed by an elevation of IL-8 levels that reached its peak at 3 μM EIPA. At higher EIPA concentrations, the curve showed another downward turn. However, IL-8 levels at 50 μM EIPA were still higher than those at 300 nM. The inhibitory effect of MIA or EIPA was not due to a decrease in cell viability, because neither of these agents decreased mitochondrial respiration at the concentrations tested (data not shown).

To further corroborate a role for NHEs in the regulation of IL-8 production, we next determined the effect of a series of NHE inhibitors that are structurally unrelated to amiloride on IL-1β-induced IL-8 produc-

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**Fig. 1.** Amiloride suppresses interleukin-1β (IL-1β)-induced IL-8 production by both HT-29 (A) and Caco-2 cells (C). Amiloride does not affect cell viability of either HT-29 (B) or Caco-2 cells (D). Amiloride was added to the cells 30 min before stimulation with IL-1β. Supernatants for IL-8 measurement were taken 24 h after the IL-1β challenge. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD550, optical density measurement at 550 nm. Data are means ± SE of n = 6 wells. *P < 0.05; **P < 0.01.

**Fig. 2.** Amiloride suppresses IL-1β-induced IL-8 production in HT-29 cells in a time-dependent manner. Amiloride (300 μM) was administered 30 min before, concurrent with, or at 0.5, 1.5, 3, 4.5, 6, 7.5, or 9 h after stimulation with IL-1β. Supernatants for IL-8 measurement were taken 24 h after the IL-1β challenge. Filled bars, control; open bars, amiloride. Data are means ± SE of n = 6 wells. *P < 0.05; **P < 0.01.
tion by HT-29 cells. The NHE inhibitors cimetidine, clonidine, and harmaline decreased IL-1β-induced IL-8 production by HT-29 cells (Fig. 3, C, D, and E). The effect of all three nonamiloride NHE inhibitors was concentration dependent (Fig. 3, C, D, and E). Under the conditions studied, none of these agents decreased cell viability at the concentrations tested (data not shown). These data confirm that NHE inhibition attenuates IL-8 production by HT-29 cells.

Finally, we tested whether amiloride inhibited the production of IL-8 induced by LPS or TNF-α. Similar to our findings with IL-1β-induced IL-8 production, amiloride pretreatment of HT-29 cells attenuated LPS- or TNF-α-induced IL-8 production (Fig. 4). In addition to suppressing cytokine- or LPS-induced IL-8 production, amiloride also decreased basal, unstimulated IL-8 production (data not shown).

**Amiloride Suppresses IL-1β-Induced IL-8 mRNA Accumulation in IECs**

To determine whether the suppressive effect of NHE inhibition on IL-8 protein production was associated with an inhibitory effect on IL-8 mRNA accumulation, we examined the effect of amiloride on IL-1β-induced accumulation of IL-8 mRNA. To this end, HT-29 cells were pretreated with amiloride (300 μM) or its vehicle (0.5% DMSO) for 30 min, which was followed by a 3 h exposure to IL-1β. At the end of the incubation period, total cellular RNA was extracted and IL-8 mRNA levels were determined by semiquantitative RT-PCR. As shown in Fig. 5, the IL-1β-induced increase in IL-8 mRNA level was prevented by pretreatment of the cells with amiloride. Amiloride also suppressed IL-1β-induced IL-8 mRNA levels in Caco-2 cells (data not shown). The effect of amiloride was not due to a general inhibition of gene transcription, because amiloride did not affect mRNA levels of the housekeeping gene GAPDH (Fig. 5). Taken together, these data suggest that the effect of amiloride on IL-8 production is pretranslational.

**NHE Blockade Inhibits IL-1β-Induced NF-κB Activation and Extracellular Signal-Regulated Kinase Phosphorylation**

Because the effect of amiloride was pretranslational, we hypothesized that amiloride may have decreased the rate of transcription due to an effect on transcription factor activation. Because NF-κB is one of the most important transcription factors that mediates the transcription of the IL-8 gene in response to IL-1β, we examined whether amiloride altered the binding of NF-κB to its consensus site using the gel mobility shift assay. Using nuclear extracts from HT-29 cells treated with IL-1β, we observed an increase in NF-κB binding compared with IL-1β-un-treated cells (Fig. 6, A and B). Supershift studies...
confirmed the observation by previous reports (2, 20) that the slower migrating complex induced by IL-1β contained both p65 and p50, whereas the faster-moving, constitutive complex contained only p50. This is because both p50 and p65 antibodies completely inhibited formation of the slower moving complex, whereas the density of the faster moving complex was clearly decreased using the p50 but not the p65 antibody (Fig. 6, A and B). The increase in NF-κB binding caused by IL-1β stimulation was partially prevented when the cells were pretreated with amiloride 30 min before IL-1β treatment. To further confirm that NHE inhibition attenuates NF-κB activation, we examined the effect of harmaline, cimetidine, and clodinine on IL-1β-induced NF-κB DNA binding. Figure 6C demonstrates that all three NHE inhibitors suppressed NF-κB activation. Collectively, these results suggest that the mechanism of action of NHE blockade in suppressing IL-8 production involves interference with the NF-κB pathway.

Next, we investigated whether the decrease in NF-κB activation corresponded with a decrease in NF-κB-dependent gene transcription. To this end, HT-29 cells were transiently transfected with a NF-κB-luciferase reporter construct. The transfectants were pretreated with amiloride or its vehicle for 30 min and then stimulated with IL-1β for 16 h. The effect of amiloride on NF-κB-dependent gene transcription was assessed using the luciferase assay. Similar to its effect on NF-κB DNA binding, amiloride suppressed IL-1β-stimulated NF-κB-dependent gene transcription (Fig. 7A). This effect was specific, because amiloride did not decrease luciferase activity when the cells were transfected with an enhancerless empty vector (data not shown). To further support the notion that the reduction of NF-κB activation by NHE inhibition is an important mechanism whereby NHE inhibition reduces IL-8 production, we conducted further pharmacological experiments using the NF-κB inhibitor pyrrolidinedithiocarbamate (59). Figure 7B shows that pyrrolidinedithiocarbamate decreased, in a concentration-dependent manner, the release of IL-8 by IL-1β-stimulated HT-29 cells, corroborating the idea that NF-κB is a pivotal inducer of IL-8 production in this system. Pyrrolidinedithiocarbamate was not toxic to the cells as measured using the MTT assay (data not shown).

Activation of extracellular signal-regulated kinase (ERK) 1/2 is an important step in the cascade of cellular events leading to IL-8 production in IECs (18, 23). Because amiloride has been demonstrated (3) to regulate MAPK activation, we evaluated whether the amiloride suppression of IL-8 production could be explained by an effect on this pathway. To this end, we pretreated HT-29 cells with amiloride or vehicle, and 30 min later the cells were stimulated with IL-1β. Whole cell extracts were prepared 30 min after the IL-1β challenge, and subsequently the activation of ERK1/2 was determined by Western blotting using an antibody against the active, phosphorylated form of ERK1/2. As shown in Fig. 8A, IL-1β increased the activation of ERK1/2 compared with IL-1β-untreated controls, and pretreatment of the cells with amiloride blunted this increase. Finally, to further ascertain that a decrease in ERK1/2 activation could contribute to the inhibitory effect of NHE blockade on IL-8 production, we tested whether ERK1/2 blockade by pharmacological means prevented IL-8 production by IL-1β-induced HT-29 cells. Figure 8B demonstrates that exposure of HT-29 cells to a selective inhibitor of the

Fig. 4. Amiloride inhibits IL-8 production induced by either tumor necrosis factor-α (TNF-α) (A) or lipopolysaccharide (LPS) (B) in HT-29 cells. Amiloride was added to the cells 30 min before stimulation with TNF-α or LPS. Supernatants for IL-8 measurement were taken 24 h after stimulation. Data are means ± SE. *P < 0.05; **P < 0.01.

Fig. 5. Amiloride pretreatment (300 μM) inhibits IL-1β-induced IL-8 mRNA accumulation in HT-29 cells. Lanes 1 and 2, control; lanes 3 and 4, IL-1β (20 ng/ml); lanes 5 and 6, amiloride + IL-1β. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were not affected by IL-1β and amiloride treatment. IL-8 and GAPDH mRNA levels were quantitated using semiquantitative RT-PCR. Values represent 3 separate experiments.
ERK1/2 pathway (PD-98059) suppresses the production of IL-8. PD-98059 did not cause any toxicity as determined using the MTT assay (data not shown).

Taken together, these observations suggest that the decreased activation of ERK1/2 may represent an important mechanism by which NHE inhibition attenuates IL-8 production.

Amiloride Attenuates Course of Colitis in DSS-Treated Mice

Administration of DSS in the drinking water induced marked colonic inflammation, as evidenced by significant weight loss (Table 1), colonic shortening (Table 1), and histological injury, as well as increased MIP-2, MPO, and MDA levels in colonic samples (Fig. 9). Treatment with amiloride (10 mg/kg) induced significant protection against these alterations (Table 1 and Fig. 9).

DISCUSSION

The activation of NHEs by inflammatory stimuli crucially contributes to inflammatory gene expression in professional inflammatory cells, such as monocytes (39) and macrophages (35, 41). The increased activity of NHEs promotes several macrophage functions, including TNF-α and IL-8 production (38, 39, 44), prostaglandin release (10), upregulation of Ia expression (41), and Fcγ receptor expression (4). There is also evidence that NHE activation is involved in promoting neutrophil migration (45) as well as MPO activity and release (53). We (35) recently demonstrated that NHE inhibition by a variety of amiloride analogs suppressed IL-12, MIP-1α, and MIP-2 production by LPS-stimulated macrophages. Furthermore, amiloride decreases IL-8 and IL-6 production in respiratory epithelium infected with respiratory syncytial virus (32). The results presented in the current study demonstrate, for the first time, that NHEs are involved in the regulation of the intestinal epithelial inflammatory response. Although amiloride is a relatively selective inhibitor of NHEs, it has effects on a number of other systems, including epithelial Na⁺ channels and the Na⁺/Ca⁺ exchanger (21, 40, 46). However, the fact that the selective NHE inhibitors MIA (IC₅₀, ~10 nM) and EIPA (IC₅₀, ~300 nM) mimicked the inhibitory effect of amiloride on IL-8...
production suggests that the primary target of amiloride in decreasing IL-8 production is the NHE. This idea was further confirmed by using the structurally different NHE inhibitors clonidine, harmaline, and cimetidine, because all three inhibitors suppressed IL-1β-induced IL-8 production. It is important to note that we (34) recently found that lithium induced both NF-κB activation and IL-8 production in human IECs. We (34) demonstrated that lithium, which is an agent known to stimulate NHEs, induced the intestinal epithelial inflammatory response. This lithium-induced intestinal cell inflammatory response is suppressed by the same NHE inhibitors as the IL-1β-induced response. The potency of these inhibitors in reducing lithium-induced IL-8 production is similar to the potency of these inhibitors in decreasing IL-1β-induced IL-8 production. Thus these data with lithium further highlight the importance of NHEs in the regulation of the IEC inflammatory response.

As described above, IECs have been shown to express NHE1, NHE2, NHE3, and NHE4; however, the expression of these isotypes varies even between different cell clones (14, 22, 31, 51). The different isotypes have differing sensitivities to inhibition by amiloride and its analogs. At this point, it would be premature to speculate which isoforms mediate the suppressive effect of NHE inhibition on IL-8 production. The high potency of EIPA and MIA is consistent with a possible role for NHE1 or NHE2. However, this picture is complicated by the fact that amiloride was less potent in decreasing IL-8 production than would have been expected. That is, the IC50 of amiloride’s suppressive effect was 30 μM, whereas amiloride has been documented to block NHE1 or NHE2 at 1 μM (50, 56). A further complicating factor is that both MIA and EIPA lost their suppressive effects in the micromolar range. We believe that while the inhibitory effect of both MIA and EIPA in the micromolar range reflects their ability to block NHEs, the reversal of inhibition in the micromolar range may be a nonspecific action. In any case, further studies are needed to characterize the NHE isoform(s) involved in the regulation of epithelial cell inflammatory responses.

Our data documenting that amiloride suppresses IL-8 mRNA accumulation are in agreement with the results of previous studies, which showed that amiloride suppressed IL-8 mRNA accumulation in human

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Table 1. Effect of amiloride on acute colon inflammation induced by DSS in male BALB/c mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Decrease in Body Weight, %</th>
<th>Colon Length, cm</th>
<th>Gross Score (median)</th>
<th>Bleeding Incidence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>−7.0 ± 3.1</td>
<td>6.1 ± 0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DSS + vehicle</td>
<td>27.1 ± 2.0*</td>
<td>3.5 ± 0.2*</td>
<td>2.5*</td>
<td>100*</td>
</tr>
<tr>
<td>DSS + amiloride</td>
<td>10.4 ± 3.1*†</td>
<td>5.1 ± 0.3*†</td>
<td>1*†</td>
<td>25*†</td>
</tr>
</tbody>
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Values are means ± SE of n = 8 animals/group. Animals were initially weighed and body weights recorded before being exposed to the 5% dextran sodium sulfate (DSS) solution ad libitum. Amiloride (10 mg/kg 1/day) was administered orally BID starting on day 1. On day 10, the experiment was terminated and the animals reweighed and killed. The colon was dissected out and measured before samples were taken and analyzed for biochemical changes. The median gross score is given. *P < 0.05, DSS animals vs. untreated animals; †P < 0.05, amiloride-treated DSS animals vs. vehicle-treated DSS animals.

Fig. 8. A: amiloride pretreatment (300 μM) inhibits IL-1β-induced extracellular signal-regulated kinase (ERK) 1/2 activation in HT-29 cells. Lanes 1 and 2, control; lanes 3 and 4, IL-1β (20 ng/ml); lanes 5 and 6, amiloride + IL-1β. Values represent 3 separate experiments. B: pretreatment of HT-29 cells with the ERK1/2 pathway inhibitor PD-98059 30 min before stimulation with IL-1β (20 ng/ml) suppresses IL-1β-induced IL-8 release. Filled bar, cells not induced with IL-1β; open bar, cells stimulated with IL-1β. Data are means ± SE of n = 14–16 wells from 2 separate experiments. **P < 0.01.
monocytes (44) or respiratory epithelium (32). Interestingly, in addition to demonstrating that amiloride suppresses IL-8 production, both of these previous studies (32, 44) found that amiloride attenuated the production of other inflammatory cytokines, including IL-6 and TNF-α. This observation and the fact that the effect of amiloride on cytokine production is likely to be transcriptional suggest that amiloride targets an early pathway that is common to the induction of proinflammatory cytokines. Our results demonstrating that amiloride, harmaline, cimetidine, and clonidine prevent both NF-κB DNA binding and NF-κB-dependent transcriptional activity in IECs indicate that this transcription factor may be the primary mediator of the anti-inflammatory effects of NHE inhibition. This notion is supported by the findings of a recent study (16), in which amiloride blocked NF-κB activation in respiratory epithelial cells.

These findings raise an important question: how is a signal provided by the membrane protein NHE transmitted to the cytosolic protein NF-κB? One of the most intriguing possibilities is that it is the alteration of the cytoskeletal organization of actin filaments that links NHEs to NF-κB and MAPK activation. This possibility is supported by the fact that NHEs are important regulators of actin filament assembly (9, 47) and that changes in the actin microfilament system are involved in the activation of the NF-κB system (1, 58). Clearly, further studies will be necessary to delineate the mechanisms whereby NHEs couple extracellular inflammatory signals to activation of the intracellular inflammatory cascade and NF-κB.

We also provide evidence that NHE inhibition by amiloride suppresses disease activity in the mouse DSS model of IBD. This decrease in IBD activity was associated with a decrease in MIP-2 production in colonic samples. MIP-2 or its human analog IL-8 is released from numerous sources during IBD. These include IECs as well as monocytes/macrophages (12, 49). Because we have shown that NHE inhibition suppresses MIP-2/IL-8 production by both epithelial cells (the current study) and macrophages (35), we propose that NHE inhibition exerts its beneficial effect, at least in part, by inhibiting the production of MIP-2/IL-8 by a variety of inflammatory cell types. Furthermore, we speculate that the mechanism of NHE promotion of inflammatory processes may have evolved as a positive feedback signal during inflammatory cell activation, and dysregulation of NHE activation may contribute to the maintenance of inflammatory processes during inflammatory/autoimmune diseases. On the other hand, recent evidence (43) suggests that NHEs and the intestinal epithelial inflammatory response are interconnected on yet another level. That is, an interesting study by Rocha et al. (43) showed that chronic treatment with the inflammatory cytokine IFN-γ downregulated the expression of NHEs in IECs. Thus it can be proposed that the downregulation of NHE expression by inflammatory signals may serve as a protective mechanism against the NHE-amplified inflammatory response during chronic inflammatory states.

In summary, our data demonstrate that NHE inhibition has anti-inflammatory effects in the gut, suggesting that the inhibition of Na+/H+ exchange may be a therapeutic approach in IBD.

REFERENCES


![Fig. 9. Effect of amiloride in the dextran sodium sulfate (DSS) colitis model: reduction in macrophage inflammatory protein-2 (MIP-2) (A), malondialdehyde (MDA) (B), and myeloperoxidase (MPO) (C) levels in the colon of mice with an acute inflammatory response measured by 10.220.33.2 on June 17, 2017 http://ajpgi.physiology.org/ Downloaded from www.ajpgi.org]


