Differential regulation of Na\(^+/\)H\(^+\) exchange isoform activities by enteropathogenic \textit{E. coli} in human intestinal epithelial cells

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\textbf{Hecht, Gail, Kim Hodges, Ravinder K. Gill, Fely Kear, Sangeeta Tyagi, Jaleh Malakooti, Krishnamurthy Ramaswamy, and Pradeep K. Dudeja. \textit{Differential regulation of Na\(^+/\)H\(^+\) exchange isoform activities by enteropathogenic \textit{E. coli} in human intestinal epithelial cells. \textit{Am J Physiol Gastrointest Liver Physiol} 287: G370–G378, 2004. First published April 8, 2004; 10.1152/ajpgi.00432.2003.—\textit{Enteropathogenic \textit{Escherichia coli}} (EPEC) is an important \textit{Escherichia coli} strain associated with infantile diarrhea in developing countries. Given the fact that EPEC is predominantly responsible for diarrheal diseases in infants and young children, although EPEC produces characteristic attaching and effacing lesions and loss of microvilli, the pathophysiology of EPEC-associated diarrhea, particularly during early infection, remains elusive. The present studies were designed to examine the direct effects of EPEC infection on intestinal absorption via Na\(^+/\)H\(^+\) exchanger (NHE) isoforms. Caco-2 cells were infected with EPEC strain E2348/69 or nonpathogenic \textit{E. coli} HB101 for a period of 60 to 120 min. Total NHE activity was significantly increased at 60 min, reaching approximately threefold increase after 90 min of EPEC infection. Similar findings were seen in HT-29 cells and T84 cells indicating that the response was not cell-line specific. Most surprising was the differential regulation of NHE2 and NHE3 by EPEC. Marked activation of NHE2 (300\%) occurred, whereas significant inhibition (~50\%) of NHE3 activity was induced. The activity of basolateral isoform NHE1 was also significantly increased in response to EPEC infection. Mutations that disrupted the type III secretion system (TTSS) ablated the effect of EPEC on the activity of both NHE2 and NHE3. These results suggest that EPEC, through a TTSS-dependent mechanism, exerts differential effects on NHE isoform activity in intestinal epithelial cells. Additionally, NHEs do not appear to play any role in EPEC-mediated inflammation, because the NHE inhibitors amiloride and 5-(N-ethyl-N-isopropyl)amiloride did not prevent EPEC-mediated I\(^{-}\)/H\(^{+}\) degradation.

\textit{Enteropathogenic \textit{Escherichia coli}} (EPEC) is a member of a group of bacteria known for their ability to produce attaching and effacing (A/E) lesions. A/E lesions are characterized by the close attachment of bacteria to host cells, resulting in formation of actin-rich protrusions, known as pedestals, and loss of surrounding microvilli. EPEC is predominantly responsible for infantile diarrhea in developing countries. Given the fact that EPEC is noninvasive and does not produce toxins, the complete mechanism(s) of diarrhea have yet to be elucidated. EPEC-mediated diarrhea appears to be multifactorial with contributing factors including disruption of tight junctions (38), inflammation (35), and concomitant neutrophil transmigration (34) that results in the release of S'-AMP, a precursor to the secretagogue adenosine (25). Additionally, NF-\(\kappa\)B activation leads to increased expression of galanin-1, which increases C\(^{-}\) secretion and fluid accumulation in the colon (17, 26).

Many enteric pathogens are known to induce diarrhea through alterations in ion transport. For example, the increase in C\(^{-}\) secretion seen in \textit{Vibrio cholerae} infection is mediated via increased levels of cAMP and activation of the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel. EPEC has been shown to have little impact on C\(^{-}\) secretion (5, 16). However, Na\(^{+}\) transport is also important in maintaining proper fluid balance in the intestine. The focus of this study was the effect of EPEC on Na\(^{+}\) transport via Na\(^+/\)H\(^+\) exchangers (NHEs). NHEs are a family of membrane proteins that carry out the electroneutral exchange of extracellular Na\(^{+}\) with intracellular H\(^{+}\) with a stoichiometry of 1:1. Of the eight isoforms of mammalian NHEs cloned to date, intestinal tissues predominantly express NHE1, NHE2, and NHE3. The ubiquitously expressed NHE1 isoform is localized to basolateral membranes and plays an important role in housekeeping functions, such as regulation of intracellular pH and cell volume. NHE2 and NHE3 isoforms are localized to the apical membrane, and it has been suggested that they mediate the vectorial absorption of Na\(^{+}\) across the intestine (40, 43). However, NHE3 has been shown to be the predominant Na\(^{+}\)-absorbing isoform in mammalian small intestine (37). Diarrhea associated with infection by enteric pathogens could result from either increased chloride secretion, decreased NaCl absorption, or both. In this regard, our previous studies (16) suggested that EPEC infection of cultured human colonic T84 cells attenuated secretagogue-induced net ion transport but had no effect on chloride secretion. The direct effects of EPEC infection on intestinal Na\(^{+}\) absorption have not been fully defined. In light of these data, we planned to directly examine the effects of EPEC infection on NHE activity in vitro using polarized human intestinal cells in culture and a commonly used, clinical isolate of EPEC strain E2348/69.

Recent studies have also implicated NHEs in the development of inflammatory response. These studies showed that the NHE inhibitor amiloride reduced IL-8 production in response to IL-1\(\beta\) (32). Additionally, LPS-mediated I\(^{-}\)/H\(^{+}\) degradation was prevented with amiloride pretreatment (31). Because inflammation is associated with EPEC infection, experiments were also performed to delineate the potential link between NHE activity and EPEC-mediated inflammation.

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Although the mechanism of EPEC-associated diarrhea is currently unknown, several advances have been made in understanding this process. For example, paracellular permeability has been shown to be disrupted by a number of signaling events (38). Alterations of tight junctions are dependent on a pathogenicity island known as the locus of enteroefface-ment (LEE) (27). The LEE encodes for a type III secretion system (TTSS) and several secreted effectors. The TTSS apparatus is a complex multiprotein structure that delivers prokaryotic molecules directly into eukaryotic host cells. The terminal structures of this apparatus consist of \textit{E. coli}-secreted proteins (Esp) EspA, EspB, and EspD. EspA forms a needle-like channel through which other secreted proteins pass (6). EspB and EspD cap this structure and together form a pore that allows direct translocation of secreted effector molecules into the host cytosol.

There are a number of described EPEC-secreted effector proteins including EspF, EspG, EspH, translocated intimin receptor (Tir), and mitochondrial associated protein (Map). EspB has also been shown to be secreted into the host cytosol; however, it is unclear whether EspB serves any function in addition to its role in pore formation. These effectors are involved in a variety of functions including pedestal formation, tight junction disruption, and impairment of mitochondrial function (13, 21). All of these responses are simultaneously lost by a number of characterized type III secretion mutations. For example, deletion mutation of either the \textit{espB} or \textit{espD} pore-forming protein gene attenuates the secretion of the five known effectors mentioned above. Due to the complexity of the type III secretion apparatus, there are a number of mutations that are capable of disrupting the translocation of the effector molecules, including \textit{E. coli} secretion component \textit{N} (esc\textit{N}). \textit{EscN} is the putative ATPase that drives the entire type III secretion process, and mutation of \textit{escN} traps all secreted molecules, including EspB and EspD, in the bacterial cytoplasm (9).

In this study, we examined the effects of EPEC infection on NHE isoform activities. The effects of a number of mutant strains were also assessed. Our results demonstrate that EPEC infection has different effects on the NHE isoforms found in intestinal epithelia; EPEC stimulated apical NHE2 and basolateral NHE1 activity, whereas NHE3 activity was significantly inhibited. Disruption of the TTSS ablated the effect of EPEC on NHE activity. Despite previously published work demonstrating a role for NHEs in the IL-1β- or LPS-mediated inflammatory response (31, 32), inhibition of these transporters had no effect on the EPEC-induced inflammatory response.

**MATERIALS AND METHODS**

**Materials.** Radionuclide \( ^{22}\text{Na}^+ \) was obtained from New England Nuclear Life Science Products (Boston, MA). Caco-2 cells and HT-29 cells were obtained from American Type Culture Collection (Manassas, VA). T84 cells were obtained from Dr. Kim Barrett (University of California, San Diego, CA). Caco-2 BBE subclone E cells were obtained from Dr. Jerrold Turner (University of Chicago, Chicago, IL). HOE-694 was a generous gift from Dr. Hans-J. Lang (Aventis, Frankfurt, Germany). Primary anti-IgBu rabbit polyclonal antibody was procured from Santa Cruz Biotechnology (lot B221; Santa Cruz, CA). All other chemicals were of at least reagent grade and were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

**Cell culture.** Caco-2 cells were grown in T-75-cm\(^2\) plastic flasks at 37°C in a 5% CO\(_2\) environment. The culture medium consisted of high-glucose DMEM, 20% fetal bovine serum, 20 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells used for these studies were between passages 25 and 42 and were plated on 24-well plates at a density of 2 × 10\(^4\) cells/well. Cells were used for experiments at days 10–14 postplating. Caco-2 BBE subclone E cells were grown exactly the same way as Caco-2 cells. HT-29 cells were grown, plated, and utilized as described for Caco-2 cells. T84 cells were grown from Santa Cruz Biotechnology or Dulbecco-Vogt modified Eagle’s medium and Ham’s F-12 medium supplemented with 6% newborn calf serum, 14 mM NaHCO\(_3\), 15 mM HEPES, 65 IU/ml penicillin, 8 μg/ml ampicillin, and 60 μg/ml streptomycin in a 5% CO\(_2\) atmosphere at 37°C. Cells were plated at a density of 8 × 10\(^4\) cells/well and required a minimum of 1 wk to reach confluence. Cells were used for experiments at 2–3 wk after plating.

**Bacterial culture and cell infection.** The following EPEC strains were used: wild-type EPEC strain E2348/69, CV2452 (E2348/69 escN::Km) (20), UMD864 (E2348/69 Δ48–759 espB1) (7), UMD870 [E2348/69 espD1::aph-3(Km)] (23), and a nonpathogenic isolate (HB101). Strains were grown overnight in the presence of appropriate antibiotics. On the day of experimentation, 30 μl of bacterial culture was transferred to 1 ml of serum- and antibiotic-free T84 cell culture medium supplemented with 0.5% mannose. Bacteria were grown ~3 h to a 0.4 OD\(_{490}\). Cell monolayers were infected at a multiplicity of infection of 100. Nonadherent bacteria were removed by washing in PBS after 30–90 min.

**Assay of NHE.** The activity of NHE was determined in acid-loaded Caco-2 cells as 5-(N-ethyl-N-isopropyl)amiloride (EIPA)-sensitive \( ^{22}\text{Na}^+ \) uptake as previously described (11). The activity of NHE isoforms was measured by using EIPA (50 μM) and HOE-694 (NHE2 isoform-specific inhibitor at 50 μM concentration). The activity of NHE2 was calculated as NHE activity sensitive to 50 μM HOE-694. NHE3 activity was calculated by subtracting the 50 μM HOE-694-sensitive NHE activity from the total NHE activity (50 μM EIPA-sensitive NHE activity). For these studies, the cells were first allowed to equilibrate by placing them at room temperature for a period of 15–20 min before the \( ^{22}\text{Na}^+ \) uptake was carried out. Briefly, confluent cell monolayers were preincubated for 30 min at room temperature in acid-load solution containing (in mM) 50 NH\(_4\)Cl, 70 choline chloride, 5 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 5 glucose, and 15 MOPS (pH 7.0). Cells were then washed with a solution containing 120 mM choline chloride and 15 mM Tris-HEPES, pH 7.5. The above solution was then aspirated, and the cells were incubated in uptake buffer containing (in mM) 3 NaCl, 110 choline Cl, 1 MgCl\(_2\), 2 CaCl\(_2\), 20 HEPES (pH 7.4), and 1 μCl/ml of \( ^{22}\text{Na}^- \), with or without 50 μM EIPA or 50 μM HOE-694. After 5 min, the \( ^{22}\text{Na}^- \)-containing uptake solution was aspirated, and the cells were washed twice with ice-cold PBS. Cells were then solubilized by incubation with 0.5 N NaOH for at least 4 h, and incorporated radioactivity was determined. The protein content of cell lysates was estimated by the Bradford method (2). \( ^{22}\text{Na}^+ \) uptake was measured at 5 min (because this was in the linear range of time course) and expressed as nanomoles per milligram of protein per 5 min.

The NHE activity assay was also modified to perform the assay without an acid-load step. After cells were infected for 10–30 min as described, cells were rinsed once in PBS and once in a solution containing 120 mM choline chloride and 15 mM Tris-HEPES, pH 7.5. Assays were then carried out for 5 min in the uptake buffer described above. The remaining procedural details were the same as for the assay of NHE activity described above.

**Basolateral membrane \( ^{22}\text{Na}^+ \) uptake using permeable supports.** For experiments using collagen-coated permeable supports, Caco-2 cells were plated on Transwell inserts (Costar, Corning, NY) at a density of 4 × 10\(^4\) cells/Transwell and used 14 days postplating. Cells were infected with EPEC from the apical side for 30 min. Unidirec-
tional basolateral membrane $^{22}\text{Na}^+$ uptake was measured, as described for 24-well plates (33) except that $^{22}\text{Na}$ and EIPA were applied only to the basolateral surface. Incubation medium alone was applied to the apical side during this step. Data are presented as the EIPA (0.5 μM)-inhibitable component in picomoles per milligram of protein per 5 min.

**Transcellular $^{22}\text{Na}^+$ flux.** Caco-2 BBE subclone E cell monolayers were used for this assay due to enhanced transepithelial resistance. Medium in the lower reservoir of the cell supports was replaced with 1 ml of medium containing either DMSO (vehicle) or EIPA (NHE inhibitor), whereas the medium in the upper chamber was then replaced with 300 μl of medium containing 1 μCi/ml of $^{22}\text{Na}$ and either DMSO (vehicle) or EIPA (NHE inhibitor) along with EPEC where relevant. At intervals of 15 min, 100 μl of medium was removed from the lower chamber and then replaced with nonradioactive medium. This process was carried out over 1 h to give four independent assessments of flux per monolayer and the assays were carried out in triplicate. Apical-to-basal flux was calculated as $J = 1(DPM \text{ time 2}) - 0.9(DPM \text{ time 1})/DPM/\text{milliliter}(\text{upper chamber})/\text{[Na}^+]$ in micromoles per milliliter, where DPM is disintegrations per minute.

**Polyethylene glycol 6000 osmoprotection.** Caco-2 cells were infected with the same as for standard NHE activity assays except that 30 mM polyethylene glycol 6000 (PEG 6000) was added to serum-free T84 medium used to resuspend bacteria. Control cells were infected with EPEC in standard serum-free T84 medium. PEG 6000 (30 mM) was also added to the acid-load buffer.

**IkBα degradation.** Confluent Caco-2 cells on 60-mm dishes were either infected with EPEC for 30, 60 or 90 min or treated with IL-1β for 15, 30, or 45 min. Cells were coincubated with DMSO (0.1% vol/vol), 50 μM EIPA, or 300 μM amiloride. Control cells were treated with DMSO, EIPA, or amiloride for 45 or 90 min. After infection, cells were rinsed in PBS and scraped into (in mm) 50 Tris (pH 7.5), 1 EDTA, 1 EGTA, 0.5 Na$_2$VO$_4$, 50 Na$^+$ chloride, 5 Na$^+$ pyrophosphate, 10 Na$^+$ β-glycerophosphate, and 0.1% β-mercaptoethanol and 1% Triton-X 100, supplemented with protease inhibitor cocktail (Sigma-Aldrich) just before addition. Extracts were centrifuged for 15 min at 12,000 g. Protein levels were determined and 24 μg of protein were separated by SDS-PAGE and blotted on nitrocellulose membrane that was then blocked for 1 h in a PBS- and goat serum-based blocking buffer (Zymed). Primary anti-IκBα rabbit polyclonal antibody was used at 0.2 μg/ml. Secondary alkaline phosphatase-conjugated goat anti-rabbit (Sigma-Aldrich) was used at 1:1,000. Blots were developed colorimetrically.

**Statistical analysis.** Data are presented as means ± SE. Student’s $t$-test was used to compare data. Significance was defined as $P \leq 0.05$.

**RESULTS**

**EPEC activate NHE.** To investigate the effects of EPEC infection on NHE activity, Caco-2 monolayers were infected with wild-type EPEC strain E2348/69 for 3 h. After infection, NHE activity was measured as EIPA-sensitive $^{22}\text{Na}^+$ uptake. As shown in Fig. 1, EPEC infection of Caco-2 cells resulted in a significant increase in NHE activity compared with the uninfected control. No significant change was observed in NHE activity when Caco-2 cells were infected with the nonpathogenic strain of *E. coli* (HB101) for 3 h.

**Time course of effect of EPEC infection on NHE activity.** We then examined the time course of EPEC-stimulated NHE activity in Caco-2 cells. For these studies, Caco-2 monolayers were infected with EPEC for different time periods ranging from 60 to 120 min, and NHE activity was measured. Figure 2A shows that EIPA-sensitive NHE activity was significantly stimulated after 60 min of EPEC infection. Although increased activity persisted, there was a progressive decrease at 90 and 120 min compared with the maximal activity achieved at 60 min.

**Effects of EPEC on NHE activity are not cell-line specific.** To evaluate whether the effects of EPEC on NHE activity were cell-line specific, NHE activity assays were also performed on T84 and HT-29 cells infected with EPEC for different time periods. Figure 2B shows the effects of EPEC infection on HT-29 cell monolayers that lack the NHE3 isoform and express only the epithelial NHE2 and basolateral NHE1 isoforms. EPEC infection caused approximately twofold increase in NHE activity as early as 60 min (Fig. 2B). Similar results were obtained in T84 cell lines (Fig. 2C). It should be noted that almost 90% of the total $^{22}\text{Na}^+$ uptake in T84 cells was HOE-694 sensitive, indicating that similar to HT-29 cells, the predominant apical isoform in this cell line is NHE2. These results suggest that the effects of EPEC on intestinal epithelial cells are not cell-line specific and also suggest that the stimulation in apical NHE activity in response to EPEC is primarily contributed by the NHE2 isoform.

**EPEC-mediated alterations in NHE activity occur early and under physiological conditions.** Although the acid-load procedure is considered to be a standard assay condition for NHE-mediated $^{22}\text{Na}^+$ uptake, we wanted to ensure that these effects also occurred under more physiological conditions as well. Additionally, negating the 30-min acid-load step allowed us to observe the effects of EPEC on NHE activity at earlier time points. As shown in Fig. 3, a significant increase in EIPA-sensitive $^{22}\text{Na}^+$ uptake was seen as early as 20 min and reached a net increase of threefold at 30 min. Whereas total uptake was reduced compared with acid-loaded cells, the values relative to controls were equivalent between the two model systems.

**EPEC has differential effects on NHE isoforms.** It was of interest to investigate the effects of EPEC on individual NHE isoform activities in Caco-2 cells that express both the apical
isoforms NHE2 and NHE3. Amiloride analogs EIPA and HOE-694 were used to dissect the effects of EPEC on NHE2 and NHE3 activity. NHE2 was defined as 50% HOE-694-sensitive NHE activity. NHE3 was defined as 50% HOE-694-insensitive NHE. Under control conditions, 49% of total NHE was contributed by NHE2 and 51% by NHE3 (Fig. 4A). Interestingly, EPEC decreased the activity of NHE3 by 50% (P<0.05), whereas NHE2 was markedly stimulated (3-fold).

Treatment with amiloride (1 μM) at levels that inhibit NHE1 had virtually no effect on Na+ uptake when applied apically rather than basally, suggesting that there is no access of the apical incubation medium to the basally located NHE1 isoform in infected monolayers at the time points studied (data not shown). To assess the effect of EPEC on NHE1 activity, localized to the basolateral surface, experiments were performed on Caco-2 cell monolayers grown on Transwells. Cells were acidified by ammonium chloride, and 22Na+ uptake was measured as EIPA-sensitive (0.5 μM) uptake from the basolateral side. As shown in Fig. 4B, EPEC infection significantly stimulated NHE1 activity.

EPEC increases the contribution of NHEs in apical-to-basal transcellular Na+ flux. To fully address the potential pro- or antidiarrheal nature of alterations in Na+ uptake, we performed transcellular Na+ flux assays. Caco-2 BBE subclone E cells were either infected with EPEC or uninfected in the presence of either DMSO vehicle or EIPA for a period of 1 h. Flux studies were performed in the apical-to-basal direction, because the largest change in uptake was due to the apical exchanger, NHE2. Studies were performed in Caco-2 BBE subclone E cells due to their higher transepithelial resistance, thus diminishing the degree of passive paracellular flux ob-
control.

Fig. 4. A: EPEC infection stimulates NHE2 activity but diminishes that of NHE3. Caco-2 cells were infected with EPEC for 90 min. After the cells were acid loaded, Na⁺ uptake was determined in the presence of EIPA (50 μM) or HOE-694 (50 μM), representing total and NHE2-specific uptake, respectively. NHE3-dependent uptake is described as total uptake/NHE2-specific uptake. NHE2 activity is increased by threefold, whereas NHE3 activity is decreased by one-half. Results represent means ± SE of 7 separate experiments performed in triplicate. *P < 0.05 compared with control. B: EPEC increases NHE1 activity. Caco-2 cells grown on permeable supports were infected with EPEC for 60 min. Because NHE1 is restricted to the basolateral membrane, EIPA (0.5 μM)-sensitive 22Na⁺ uptake was measured from the basolateral side in the acid-loaded cells. EPEC infection stimulated NHE1 activity. Results represent means ± SE of 3 observations in triplicate. *P < 0.05 compared with control.

served in standard Caco-2 cells. We noted a small but insignificant increase in total unidirectional Na⁺ flux in EPEC-infected cells compared with uninfected cells; however, the most dramatic change was in the net contribution of NHE-mediated uptake to total apical-to-basal flux (Fig. 5). The EIPA-inhibitable portion of flux was 10-fold greater in infected cells compared with uninfected cells (0.024 and 0.0023 μM·h⁻¹·cm⁻², respectively; P < 0.01 by Student’s t-test). It appears that most of this increase in NHE activity is compensated for through the loss of some other mechanism(s) of Na⁺ uptake that remains to be defined.

EPEC-induced increases in 22Na⁺ uptake are dependent on a functional TTSS. EPEC delivers effector molecules into host cells via a TTSS. E. coli secretion component N (escN) is the putative ATPase that drives type III secretion. To determine whether a functional TTSS was required for the EPEC-associated changes in NHE activity, cells were infected with an EPEC strain harboring a mutation in the escN gene. Typically, EPEC induced a marked increase in both total and NHE2-driven 22Na⁺ uptake and a concomitant decrease in NHE3-mediated Na⁺ uptake (Fig. 4A). Rendering of the TTSS non-functional by mutating escN abrogated this effect (Fig. 6). That is, compared with uninfected control cells, there was no significant increase in NHE activity after infection with the escN mutant strain. Because it was possible that values for the individual NHE isoforms could have changed without altering the total Na⁺ uptake, HOE-694 was used to specifically inhibit NHE-2. As can be seen in Fig. 6, there was no significant change in uptake levels for either NHE2 or NHE3 when cells were infected with the escN mutant. These data suggest that the EPEC-induced attenuation in NHE activity is a type III secretion-dependent process.

Fig. 5. EPEC alters the contribution of NHEs to 22Na⁺ flux. Transepithelial 22Na⁺ flux was measured in the apical-to-basal direction in serum-free medium. We added 300 μl of 1 μCl/ml 22Na⁺ to the apical compartment of permeable supports with either DMSO or EIPA and with wild-type EPEC where relevant. The basal compartment contained 1 ml of nonradioactive medium with either DMSO or EIPA where appropriate. Samples (100 μl) of this reservoir were taken every 15 min over 1 h. Flux was calculated as described in MATERIALS AND METHODS and expressed as micromoles per hour per centimeter squared. Data are means ± SE of 5 experiments in triplicate with 4 sample points. *P < 0.05 compared with control.
To further analyze the role of the TTSS in EPEC-altered NHE activity, cells were infected with *E. coli*-secreted protein B (espB) and *E. coli*-secreted protein D (espD) mutant strains that are incapable of forming translocation pores. Infection of cells with either the espB or espD mutant strain did not significantly increase Na$^+$ uptake compared with uninfected control, suggesting that either the translocation pore itself or the secreted effectors proteins are required for the EPEC-mediated increase in $^{22}$Na$^+$ uptake (Fig. 7A).

**Increased Na$^+$ uptake levels are not due to osmotic stress.**

One of the potential mechanisms for the observed increase in $^{22}$Na$^+$ uptake could have been osmotic shock due to the pore formation process. EPEC-induced pores have been previously characterized with regard to the size of molecules that are incapable of passing through the pore (18). PEG 6000 is sufficiently large to be excluded from EPEC pores and can therefore be utilized to osmotically balance the medium (18). This same approach has been used to prevent EPEC pore-mediated lysis of red blood cells. Osmotic balancing prevents excess pressure on the eukaryotic cells and, therefore, the osmotic shock that is often associated with pore-forming toxins. As can be seen in Fig. 7B, there was a slight decrease in Na$^+$ uptake with PEG 6000 treatment; however, this difference was not significant. Therefore, the increased Na$^+$ uptake does not appear to be due to osmotic shock mediated by EPEC pores.

**Blocking NHE activity does not prevent EPEC-mediated inflammation.**

Recent studies have reported a link between the inflammatory response, including IκBα degradation, and NHE activity. We have previously reported that EPEC infection induces IκBα degradation, NF-κB activation, and IL-8 production (35, 36). We questioned, therefore, whether NHE activation by EPEC infection contributed to these events. Cells were infected with EPEC in the presence of the NHE inhibitors EIPA or amiloride. If NHE activity was involved in the EPEC-mediated inflammatory cascade, then IκBα degradation would be attenuated in the presence of NHE inhibitors. As can be seen in Fig. 8A, the degree of IκBα degradation was equivalent in cells treated with either the NHE inhibitor or the DMSO vehicle. A marked decrease in IκBα was seen at 90 min postinfection compared with the uninfected controls. Neither EIPA (data not shown) nor amiloride (Fig. 8A) altered basal levels of IκBα in the cells. As a control for this experiment, cells were treated with IL-1β, a potent mediator of IκBα degradation. IκBα degradation occurred within 15 min with levels beginning to be restored at 45 min (Fig. 8B). In our system, neither EIPA or amiloride treatment prevented IL-1β-induced degradation of IκBα.

**DISCUSSION**

The pathophysiology of EPEC-induced diarrhea remains complex, especially in the absence of typical enteroxotoxin production. The A/E phenotype, considered crucial to EPEC pathogenicity, has not been able to fully explain the underlying mechanisms that cause diarrhea. It was originally presumed that microvillus degeneration associated with A/E lesion formation could be responsible for EPEC-induced diarrhea; however, studies involving human volunteers suggested that malabsorption due to A/E lesions would not explain the early onset of diarrhea (14). The present studies clearly demonstrate that infection of Caco-2 monolayers with EPEC for 1–3 h results in a significant stimulation in NHE2 but significant inhibition of NHE3 exchange activity. The effects of EPEC on NHE activity were evident at time points as early as 20 min postinfection. The inability of the nonpathogenic *E. coli* to alter Na$^+$ transport indicates that the effects of EPEC were specific. Whereas the decrease in NHE3 activity would tend to promote diarrhea, the substantial increase in NHE2-mediated Na$^+$ uptake is expected to be anti diarrheal and may actually be compensatory in nature. The actual mechanism(s) behind EPEC-mediated diarrhea are complex and include disruption of tight junctions (38) that leads to an increase in paracellular permeability. The larger process of inflammation has several downstream effects, including an influx of neutrophils (34) that release 5'-AMP that is converted into the secretagogue adenosine (25). Furthermore, the activation of NF-κB leads to upregulation of galanin-1, which can lead to increased CΙ$^-$ secretion (17, 26). EPEC-mediated diarrhea, therefore, is a multifactorial process with alterations in Na$^+$ uptake clearly

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**Fig. 7.** EPEC pore-forming proteins EspB and EspD are necessary for alteration of NHE activity. A: Caco-2 cells were infected with wild-type EPEC, espB, or espD mutants for 30 min, rinsed with PBS, acid loaded for an additional 30 min, and then used to determine EIPA (50 μM)-sensitive Na$^+$ uptake. Na$^+$ uptake in espB and espD mutant-infected cells was not significantly different from that of uninfected control cells. Data are the means of 4 experiments in triplicate with SE. B: osmoprotection of EPEC-treated cells with polyethylene glycol 6000 (PEG 6000) has no effect on Na$^+$ uptake. To determine whether EPEC-mediated pore formation played a role in increased Na$^+$ uptake, Na$^+$ uptake by causing osmotic shock. Caco-2 cells were infected with PEG 6000 resuspended in either medium alone or containing 30 mM PEG 6000, an osmoprotectant. After 30 min, cells were rinsed, and medium was replaced with acid-load solution or acid-load solution supplemented with PEG 6000. There was a slight but insignificant reduction in Na$^+$ uptake by PEG 6000-treated, EPEC-infected cells, suggesting that osmotic shock does not play a role in increased Na$^+$ uptake. Data are the means of 2 experiments performed in triplicate with SE. *P < 0.05 by Student’s t-test.
the other hand, is an epithelial cell line derived from colon carcinoma that has been shown to predominantly express NHE2 and lack the epithelial NHE3 isoform (12). We elected to use Caco-2 cell as a model system to investigate the individual molecular isoforms of NHEs regulated by EPEC.

Previous studies from our laboratory (1, 10, 11) and others (19, 28, 30, 42) have provided substantial evidence for the suitability of Caco-2 cells in exploring the functions of NHE isoforms. A number of reports have demonstrated the acute regulation of the apical NHE isoforms NHE2 and NHE3 and, in some cases, these isoforms are known to be differentially regulated. In C2/bbe cells, for example, both isoforms are downregulated by cAMP; however, in the C2/bbe cell line, NHE2 is upregulated by serum and unaffected by guanosine 3',5'-cyclic monophosphate (cGMP) or PKC, whereas NHE3 is downregulated by cGMP and PKC (28). We previously reported (11) the inhibition of NHE3 by nitric oxide in Caco-2 cells with no effects on NHE2 activity. More recently, we (10) demonstrated that serotonin inhibits both NHE2 and NHE3 activity with more pronounced effects on NHE2. Interestingly, our present studies indicate the differential regulation of NHE2 and NHE3 by EPEC, where NHE3 activity is defined as EIPA-sensitive \( \text{Na}^+ \) uptake minus uptake in the presence of the NHE2-specific inhibitor HOE-694 (50 \( \mu \)M). Our data show that the stimulation of \( \text{Na}^+ \) uptake in response to EPEC was contributed mainly by NHE2 activation. However, the significant decrease (50%) in NHE3 activity, which was masked by the presence of dramatically increased NHE2 activity, could imply an important role for NHE3 in regulating \( \text{Na}^+ \) absorption in the intestine and its pathophysiological role in diarrhea. This statement may be particularly relevant because basal fluid absorption by the intestine was severely diminished in NHE3 knockout mice; however, NHE2 knockout mice had no intestinal absorption deficit (37). Also, Ledoussal et al. (24) showed that NHE2 activity does not compensate for diarrhea produced in NHE3 knockout mice. Therefore, inhibition of NHE3 may make a significant contribution to the development of diarrhea.

It is possible that modulations in NHE activity are linked to epithelial permeability changes caused by EPEC. In this regard, previous studies (4, 5) have shown a reduction in the transepithelial electrical resistance of intestinal epithelial monolayers infected with EPEC. However, this possibility seems unlikely in our present studies, because NHE2 and NHE3 are differentially regulated in this system and ion exchange that is compensating for increased paracellular ion flux should be unidirectional. Additionally, whereas transepithelial resistance (TER) reductions are possible during the longer Caco-2 cell infections (4, 5), it seems unlikely that the data presented for T84 cells are subject to such speculation, because we have previously shown no significant changes in TER until 3 h of infection in confluent monolayers of T84 cells (16). It could also be argued that the observed effects of EPEC on NHE2 and NHE3 activity are secondary to changes in the activity of the basolateral NHE1 isoform. Our data using permeable supports showed that EPEC infection significantly stimulated basolateral NHE1 activity. Because EPEC induced a differential response of NHE3 and NHE2, it would suggest that these NHE changes are not secondary to alterations in the \( \text{Na}^+ \) concentration or intracellular pH (pHi) changes caused by activation of NHE1, which would have resulted in decreased activity of both NHE2 and NHE3. Because EPEC decreases
the pH of the growth medium, an alteration in the pH, concentration of cells, a driving force for changes in NHE activity, should be considered. There are, however, several arguments that rule out the pH-dependent NHE activity changes. First, pH was monitored throughout the course of an infection and did not change until 2 h of infection (data not shown). Because all of the infections described here involved a maximum of 90 min of infection before a rinse step and the majority utilized only a 30-min infection procedure before acid load, the potential pH changes could not explain NHE changes observed. Additionally, non-pathogenic and type III-defective EPEC mutants share the same metabolism as wild-type EPEC and therefore also alter pH accordingly; however, these strains did not increase Na\(^+\) uptake. Furthermore, pH changes would not be responsible for differential regulation of NHE isoforms, i.e., increase in NHE2 and NHE1 and a decrease in NHE3 activity.

Another mechanism that was considered to underlie the changes in NHE activity was hyperosmolarity. The pattern of NHE1 and NHE2 uptake increase with concomitant NHE3 decrease is typically indicative of either hyperosmolarity-related changes or PKC activation. However, the inability of either non-pathogenic *E. coli* HB101 or type III secretion mutants *escN*, *espB*, and *espD* to increase Na\(^+\) uptake indicates that this response is not due to hyperosmolarity but instead suggests possible signaling events induced by EPEC. At the most basic level, the increase in Na\(^+\) uptake could be attributed to either the type III secretion apparatus itself or the effector molecules secreted into host cells. Pore formation in the host cell membrane could be responsible for changes in Na\(^+\) uptake, because pore-forming toxins are known to induce osmotic stress on host cells (29). In its most extreme form, osmotic pressure can lead to cell lysis. Whereas PEG 6000 is sufficient to prevent osmotic shock, it was not sufficient to prevent changes in Na\(^+\) uptake, suggesting that EPEC-induced changes are not due to osmotic shock from pore formation. There was a slight increase in baseline Na\(^+\) uptake in the presence of PEG 6000 without EPEC, which is most likely due to the increased osmolarity of the medium.

Whereas this is the first report implicating EPEC type III secretion in NHE-mediated alteration of Na\(^+\) uptake, it is not the first report suggesting involvement of the TTSS in a more general perturbation of ion flux. Collington et al. (4) found an early and transient increase in short-circuit current of Caco-2 cells that was abrogated in *escN*, *espB*, *espD*, and *espA* mutants, all of which interfere with type III secretion. Similarly, Stein et al. (39) found that EPEC could produce a reduction in Caco-2 cell-resting membrane potential that was dependent on an intact TTSS. Although there could be potential effects of the type III secretion apparatus itself on Na\(^+\) absorption, it is far more likely that one or more of the secreted effector molecules are responsible for the increase in Na\(^+\) uptake. There are currently several predominant proteins known to be injected into the host cytosol. These include EspB, EspF, EspG, EspH, Tir, and Map, and a recent report (41) suggests that these six may be the only injected proteins in the entire LEE. These effector molecules produce a wide array of phenotypes. Perhaps the most interesting with regard to ion exchange are those produced by the effector molecule Map. Map alters the membrane potential of mitochondria within infected host cells through as yet undefined mechanisms (22).

Inflammation also plays an important role in the course of EPEC infection, and NHEs have been recently implicated in inflammation leading to IκB\(\alpha\) degradation and production of IL-8 (31, 32). Neither of the NHE inhibitor amiloride or EIPA prevented EPEC-mediated IκB\(\alpha\) degradation. As a positive control, we attempted to block IL-1β-mediated degradation of IκB\(\alpha\); however, this too proved ineffective. The individual lots of EIPA and amiloride were tested and shown to be highly effective in blocking NHE activity in \(^{22}\)Na\(^+\) uptake assays, suggesting that NHE activity is not required for IκB\(\alpha\) degradation by EPEC. The experiments performed here focus on IκB\(\alpha\) degradation rather than IL-8 production as in Nemeth et al. (32) and were simply intended to determine whether the EPEC-specific inflammatory cascade could be altered by blocking NHE activity.

In summary, we report the novel finding that EPEC infection differentially regulates the activity levels of the three intestinal NHE isoforms, NHE1, NHE2, and NHE3. Although increases in total Na\(^+\) uptake are expected to be anti-diarrheal, we believe that the observed increase in NHE2 activity might represent a potential compensatory response to increased luminal fluid resulting from attenuation of NHE3 activity, tight junctions disruption, inflammatory response, upregulation of galanin-1, or alterations in anion exchangers. In parallel studies (15), we have also shown significant (∼70%) inhibition of Cl\(^-\)/OH\(^-\) exchange activity in Caco-2 cells infected with EPEC for 60–120 min. The decrease in NHE3 activity observed in present studies and decreased Cl\(^-\)/OH\(^-\) exchange activity could, in part, contribute to the pathophysiological basis of EPEC-induced diarrhea. The data presented here also indicate that the effects of EPEC on NHE activity are dependent on a functional TTSS. Future studies will be aimed at delineating the cascade of signaling events that modulate NHE isoform activity in response to EPEC and the effector molecules responsible.

**REFERENCES**

REGULATION OF Na⁺ TRANSPORT BY EPEC


