Prostaglandin-mediated inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchanger isoform 2 stimulates recovery of barrier function in ischemia-injured intestine

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Submitted 22 March 2006; accepted in final form 27 April 2006

Moeser, Adam J., Prashant K. Nighot, Kathleen A. Ryan, Jenna G. Wooten, and Anthony T. Blikslager. Prostaglandin-mediated inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchanger isoform 2 stimulates recovery of barrier function in ischemia-injured intestine. *Am J Physiol Gastrointest Liver Physiol* 291: G885–G894, 2006. First published March 30, 2006; doi:10.1152/ajpgi.00380.2005.—Prostaglandins stimulate repair of the ischemia-injured intestinal barrier in the porcine ileum through a mechanism involving cAMP-dependent Cl\textsuperscript{−} secretion and inhibition of electroneutral Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) activity. In the present study, we focused on the role of individual NHE isoforms in the recovery of barrier function. Ischemia-injured porcine ileal mucosa was mounted on Ussing chambers. Short-circuit current (I\textsubscript{sc}), transepithelial electrical resistance (TER), and isotopic fluxes of \(^{22}\text{Na}\) were measured in response to PGF\textsubscript{2} and selective inhibitors of epithelial NHE isoforms. Immunoassays were used to assess the expression of NHE isoforms. Forty-five minutes of intestinal ischemia resulted in a 45\% reduction in TER (P < 0.01). Near-complete restitution occurred within 60 min. Inhibition of NHE2 with HOE-694 (25 \(\mu\text{M}\)) added to the mucosal surface of the injured ileum stimulated significant elevations in TER, independent of changes in I\textsubscript{sc} and histological evidence of restitution. Pharmacological inhibition of NHE3 or NHE1 with mucosal S-3226 (20 \(\mu\text{M}\)) or serosal cariporide (25 \(\mu\text{M}\)), respectively, had no effect. Ischemia-injured tissues treated with mucosal S-3226 or HOE-694 exhibited equivalent reductions in mucosal-to-serosal fluxes of \(^{22}\text{Na}\) (by \(\sim 35\%\)) compared with nontreated ischemia-injured control tissues (P < 0.05). Intestinal ischemia resulted in increased expression of the cytoplasmic NHE regulatory factor EBP\textsubscript{50} in NHE2 but not in NHE3 immunoprecipitates. Selective inhibition of NHE2, and not NHE3, induces recovery of barrier function in the ischemia-injured intestine.

The gastrointestinal tract is lined by a single layer of epithelium, which forms a highly selective barrier designed to allow the efficient transport of nutrients and water while preventing the entry of potentially toxic luminal pathogenic organisms and their products. Breakdown of the intestinal barrier is characterized by increased intestinal permeability, which precedes the onset of a number of acute and chronic intestinal disorders including inflammatory bowel diseases, celiac disease, and, in extreme cases, multiple organ dysfunction syndrome, the leading cause of morbidity and mortality in intensive care unit patients (11, 25, 41). Utilizing a porcine model of intestinal ischemia, we have accumulated evidence indicating that prostaglandins (PGs) orchestrate repair of intestinal barrier function within restitution of epithelium. Ussing chamber experiments have demonstrated that blockade of endogenous PG synthesis with the nonselective, nonsteroidal anti-inflammatory drug indomethacin impairs the ability of the intestine to recover barrier function as assessed by transepithelial electrical resistance (TER). Moreover, application of PG analogs (PGE\textsubscript{2} and PGI\textsubscript{2}) to the ischemia-injured mucosa resulted in the rapid restoration of TER values to uninjured control levels (4–6). Histological evaluation of recovering ischemia-injured tissues showed that the reparative events mediated by PGs in these studies appear to be related to permeability changes in the tight junctions and paracellular space rather than enhanced rates of epithelial restitution (5, 16).

A number of prostanoids signal via cAMP, leading to stimulation of apical Cl\textsuperscript{−} secretion and inhibition of electroneutral Na\textsuperscript{+} absorption, which are important mechanisms in the pathogenesis of diarrhea (1, 13, 18, 34). Paradoxically, these same alterations in electrolyte transport are critical processes in the repair of intestinal barrier function following ischemic injury. Although secretory processes associated with diarrhea are predominantly a result of Cl\textsuperscript{−} and HCO\textsubscript{3}\textsuperscript{−} secretion through CFTR channels in the intestinal crypts, recent evidence suggests that the Cl\textsuperscript{−}-dependent reparative role of PGs occurs in the tight junctions of the villus epithelium through targeted activation of CIC-2 Cl\textsuperscript{−} channels (29). The electroneutral Na\textsuperscript{+}-dependent processes involved in intestinal barrier repair are unclear and require further investigation.

To date, there are nine identified Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) isoforms, of which NHE1–3 appear to be the major intestinal isoforms (50). In most mammals, NHE3 is the predominant brush-border NHE contributing to electroneutral Na\textsuperscript{+} absorption, whereas NHE2, which is also expressed apically, contributes to a lesser degree in the small intestine (15). For example, mice with genetic modifications in NHE isoforms exhibit diarrhea in the absence of NHE3 but no signs of diarrheal disease in the absence of NHE2 (22, 37). The ubiquitously expressed NHE1 isoform is present on the basolateral surface of cells and functions in pH and volume regulation (14, 35).

Studies have shown that inhibition of apical NHE activity can modulate changes in intestinal permeability via interactions with intracellular regulatory proteins, although these pathways are as yet unclear (42, 44). We have demonstrated an important role of inhibition of apical electroneutral Na\textsuperscript{+}/H\textsuperscript{+} exchange in PG-stimulated recovery of barrier function in the ischemia-injured intestine. A contractile response of the intestine occurs in the ischemia-injured intestine. A contractile response of the intestine occurs in the ischemia-injured intestine.
ischemia-injured intestine; however, the contributory roles of individual NHE isofoms in barrier repair are unknown. The objective of this study was to investigate the role of electroneutral Na\(^+\) absorption in the recovery of intestinal barrier function, specifically addressing the role of individual NHE isofoms in the recovering ischemia-injured mucosa.

MATERIALS AND METHODS

Experimental porcine surgeries. All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Six- to eight-week-old Yorkshire crossbred pigs of either sex were housed singularly and maintained on a commercially pelleted feed. Pigs were held off feed for 12 h prior to experimental surgery. General anesthesia was induced with xylazine (1.5 mg/kg im), ketamine (11 mg/kg im), and thiopental (15 mg/kg iv) and was maintained with an intermittent infusion of thiopental (6–8 mg\(\cdot\)kg\(^{-1}\)\cdot h\(^{-1}\)). Pigs were placed on a heating pad and ventilated with 100% O\(_2\) via a tracheotomy using a time-cycled ventilator. The jugular vein and carotid artery were cannulated, and blood gas analysis was performed to confirm normal pH and partial pressures of CO\(_2\) and O\(_2\). Lactated Ringer solution was administered intravenously at a maintenance rate of 15 ml\(\cdot\)kg\(^{-1}\)\cdot h\(^{-1}\). The ileum was approached via a ventral midline incision. Ileal segments were delineated by ligating the intestine at 10-cm intervals and were subjected to ischemia by occluding the local mesenteric blood supply for 45 min.

Ussing chamber studies. Following the 45-min ischemic period, tissues were harvested from the pig, and the mucosa was stripped from the seromucosal layer in oxygenated (95% O\(_2\)-5% CO\(_2\)) Ringer solution (in mmol/l: 154 Na\(^+\), 6.3 K\(^+\), 137 Cl\(^-\), and 24 HCO\(_3\); pH 7.4) containing 5 μM indomethacin to prevent endogenous PG production during the stripping procedure. Tissues were then mounted in 3.14-cm\(^2\) aperture Ussing chambers, as described in a previous study (2). For each Ussing chamber experiment (n = 6), ileal tissues were mounted on multiple Ussing chambers and subjected to different in vitro treatments. Tissues were bathed on the serosal and mucosal sides with 10 ml Ringer solution. The serosal bathing solution contained 10 mM glucose and was osmotically balanced on the mucosal side with 10 mM mannitol. Bathing solutions were oxygenated (95% O\(_2\)-5% CO\(_2\)) and circulated in water-jacketed reservoirs. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short circuited through Ag-AgCl electrodes using a voltage clamp that was corrected for fluid resistance. TER (Ω\(\cdot\)cm\(^{-2}\)) was calculated from the spontaneous PD and short-circuit current (Isc). If the spontaneous PD was between −1.0 and 1.0 mV, tissues were current clamped at ±100 μA for 5 s, and the PD was recorded. Isc and PD were recorded at 15-min intervals over a 4-h experiment.

Experimental treatments. After being mounted on Ussing chambers, tissues were allowed to acclimate for a period of 30 min to achieve stable baseline measurements. For experiments assessing the role of NHE isofoms in the PGE\(_2\)-induced recovery of barrier function, tissues were pretreated (time 0) with pharmacological NHE inhibitors on the appropriate surface.

Isotopic Ussing chamber flux studies. These studies were performed at the same time as electrical measurements were recorded. To assess transmucosal Na\(^+\) fluxes, 22Na was added to the mucosal or serosal solutions of tissues paired according to their conductance (conductance within 25% of each other). Following a 15-min equilibration period and prior to the addition of treatments, standards were taken from the bathing reservoirs. Thirty minutes after the addition of treatments, three successive 60-min flux periods (from 60 to 240 min of the experiments) were performed by taking samples from the bathing reservoirs opposite to the side of isotope addition. Samples were counted for 22Na in a liquid scintillation counter. Unidirectional Na\(^+\) fluxes from the mucosa to serosa (J\(_{ms}\)) and serosa to mucosa (J\(_{sm}\)) and the net flux (J\(_{net}\)) were determined using standard equations.

Histological examination. Tissues were taken at 0, 60, and 240 min of the in vitro recovery period for routine histological evaluation. Tissues were sectioned (5 μm) and stained with hematoxylin and eosin. For each tissue, three sections were evaluated. Four well-oriented vili and crypts were identified in each section. Villus length was obtained using a micrometer in the eye piece of a light microscope. In addition, the height of the epithelium-covered portion of each villus was measured. The surface area of the villus was calculated using the formula for the surface area of a cylinder. The formula was modified by subtracting the area of the base of the villus and multiplying by a factor accounting for the variable position at which each villus was cross sectioned (2). The percentage of the villous surface area that remained denuded was calculated from the total surface area of the villus and the surface area of the villus covered by epithelium. The percent denuded villous surface area was used as an index of epithelial restitution.

Immunohistochemistry. For immunohistochemical analyses, tissues were fixed in 10% neutral buffered formalin for 24 h, transferred to a 70% ethanol solution, and embedded in paraffin. Five-micrometer sections were mounted on slides, deparaffinized, and rehydrated. Slides were subsequently incubated in 3% H\(_2\)O\(_2\), after which endogenous avidin and biotin were inhibited using an ABC staining kit (Santa Cruz Biotechnology, Santa Cruz, CA). After slides were further washed in PBS, they were incubated for 1 h at room temperature with a 1:50 dilution of either goat anti-NHE3 or anti-NHE2 polyclonal antibodies (Santa Cruz Biotechnology). After this, slides were incubated with anti-goat secondary antibody.

Gel electrophoresis and Western blot analysis. Ileal scrapings from the control and ischemia-injured mucosa were snap frozen and stored at −70°C before SDS-PAGE. Tissue aliquots were thawed at 4°C and added to 3 ml chilled lysis buffer, including protease inhibitors (0.5 mM Pefabloc, 0.1 mM 4-nitrophenyl phosphate, 0.04 mM β-glycerophosphate, 0.1 mM Na\(_3\)VO\(_4\), 40 μg/ml bestatin, 2 μg/ml aprotinin, 0.54 μg/ml leupeptin, and 0.7 μg/ml pepstatin A) at 4°C. This mixture was homogenized on ice and then centrifuged at 4°C, and the supernatant was saved. Protein analysis of extract aliquots was performed (DC protein assay; Bio-Rad, Hercules, CA). Tissue extracts (amounts equalized by protein concentration) were mixed with an equal volume of 2× SDS-PAGE sample buffer and boiled for 4 min. Lysates were loaded on a 10% SDS-polyacrylamide gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Science, Birmingham, UK) by using an electroblotting minitransfer apparatus. Membranes were blocked at room temperature for 60 min in Tris-buffered saline plus 0.05% Tween 20 and 5% dry powdered milk. Membranes were washed and then incubated for 60 min in primary antibody (rabbit anti-NHE2 and NHE3, 2 μg/ml; Chemicon). After an additional wash, membranes were incubated with horseradish peroxidase-conjugated secondary antibody and developed for visualization of protein by the addition of enhanced chemiluminescence reagent (Amersham, Piscataway, NJ).

Immune precipitation. Tissue extracts were prepared according to the Western blot analysis protocol described previously. Extracted proteins (1 ng) were solubilized in RIPA buffer [50 mM Tris•HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% (wt/vol) SDS, and 1% Triton X-100 with protease inhibitors] and incubated with either NHE2 or NHE3 anti-goat polyclonal antibodies (10 μg/ml; Santa Cruz Biotechnology) for 1 h at 37°C. The antibody-protein complexes were adsorbed from solution with protein A/G-Agarose beads (Santa Cruz Biotechnology). Protein samples were washed three times in ice-cold RIPA buffer and centrifuged at 25,000 g for 1 min at 4°C. Protein pellets were solubilized in 2× SDS-PAGE sample buffer, and electrophoresis was carried out according to standard procedures described previously. Nitrocellulose membranes were incubated with anti-NHE2 or anti-NHE3 (1:2,000 dilution). For immunoprecipitation experiments, immunoprecipitated NHE2 was subjected to SDS-
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INHIBITION OF NHE2 MEDIATES BARRIER FUNCTION RECOVERY

Blockade of apical Na\(^+\)/H\(^+\) exchange enhances recovery of TER in ischemia-injured mucosa. To confirm previous studies demonstrating the importance of electrogenic Cl\(^-\) secretion and inhibition of NHE activity in the recovery of barrier function in the ischemia-injured ileum, we mounted tissues on Ussing chambers and monitored electrophysiological parameters in response to PGE\(_2\) and inhibitors of brush-border electroneutral Na\(^+\) absorption and Cl\(^-\) uptake (Fig. 1, A and B). Ischemic injury resulted in marked reductions in TER compared with uninjured control tissue (49 ± 2.3 Ω·cm\(^2\)) in control tissue vs. 27 ± 1.6 Ω·cm\(^2\) in ischemia-injured mucosa, \(P < 0.05\)). Application of 1 μM PGE\(_2\) to the serosal surface of the ischemia-injured ileal mucosa stimulated rapid elevations in TER (ΔTER = 28 ± 3.1 Ω·cm\(^2\), \(P < 0.01\)) that attained control levels within 45 min of PGE\(_2\) application, whereas tissues treated with indomethacin had minimal elevations in TER over the 240-min recovery period. PGE\(_2\)-stimulated elevations in TER were preceded by dramatic elevations in Cl\(^-\) secretion evidenced by increased \(I_{sc}\) (Δ\(I_{sc}\) = 19 ± 3.9 MA/cm\(^2\), \(P < 0.001\)) that were inhibited by bumetanide (0.1 mM), an inhibitor of the Na\(^+\)-K\(^+\)-2Cl\(^-\) transporter, the predominant mechanism for Cl\(^-\) uptake across the basolateral membrane (Fig. 1B). Although bumetanide inhibited \(I_{sc}\) elevations in these tissues, it did not abolish elevations in TER. To assess the role of NHE inhibition in recovery of TER, we treated ischemia-injured tissues with amiloride (1 mM), an inhibitor of apical NHE activity. Amiloride induced elevations in TER that were similar in magnitude and time course to those observed in tissues treated with bumetanide, suggesting that the \(I_{sc}\)-independent events in PG-mediated repair were attributable to inhibition of NHE.

Effects of Na\(^+\)-free mucosal Ringer solution on TER in ischemia-injured porcine ileal mucosa. We performed additional experiments with ischemia-injured mucosa bathed in Na\(^+\)-free Ringer solution to assess the role of NHE inhibition on the recovery of TER. In these experiments, mucosal Na\(^+\) was replaced with D-glucamine chloride, which inhibits apical NHE function by decreasing the Na\(^+\) available for exchange (35). Mucosal Na\(^+\) substitution stimulated significant (\(P < 0.05\)) elevations in TER over ischemia-injured control tissue that were similar in magnitude and time course to ischemia-injured tissue treated with amiloride (Fig. 2).

Recovery of TER in ischemia-injured ileal mucosa is mediated by selective inhibition of NHE2. Because blockade of brush-border NHE activity with amiloride induced elevations in TER in the ischemia-injured ileal mucosa, we sought to further define this mechanism by investigating whether a particular NHE isoform was primarily responsible for increased TER. Therefore, the ischemia-injured ileal mucosa was treated with selective NHE isoform inhibitors, and TER was monitored over a 240-min period. NHE3, the predominant isoform mediating electroneutral Na\(^+\) absorption in the small intestine of most mammals (15, 28, 38), was initially assessed by treating the ischemia-injured porcine mucosa with selective NHE3 inhibitor S-3226. This compound has a reported IC\(_{50}\) for NHE3 of 0.2 μM in porcine renal brush-border membrane vesicles (39). Application of S-3226 (20 μM) to the mucosal surface of the ischemia-injured mucosa had no effect on TER. Therefore, the ischemia-injured ileal mucosa was treated with selective NHE isoform inhibitors, and TER was monitored over a 240-min period (Fig. 3). NHE3, the predominant isoform mediating electroneutral Na\(^+\) absorption in the small intestine of most mammals (15, 28, 38), was initially assessed by treating the ischemia-injured porcine mucosa with selective NHE3 inhibitor S-3226. This compound has a reported IC\(_{50}\) for NHE3 of 0.2 μM in porcine renal brush-border membrane vesicles (39). Application of S-3226 (20 μM) to the mucosal surface of the ischemia-injured mucosa had no effect on TER. Therefore, the ischemia-injured ileal mucosa was treated with selective NHE isoform inhibitors, and TER was monitored over a 240-min period (Fig. 3). NHE3, the predominant isoform mediating electroneutral Na\(^+\) absorption in the small intestine of most mammals (15, 28, 38), was initially assessed by treating the ischemia-injured porcine mucosa with selective NHE3 inhibitor S-3226. This compound has a reported IC\(_{50}\) for NHE3 of 0.2 μM in porcine renal brush-border membrane vesicles (39). Application of S-3226 (20 μM) to the mucosal surface of the ischemia-injured mucosa had no effect on TER.
on TER compared with ischemia-injured controls, suggesting an insignificant role of NHE3 inhibition in the recovery of TER in these tissues.

Given that pharmacological inhibition of NHE3 failed to induce the recovery of TER as observed with amiloride, we assessed the role of the NHE2 brush-border isoform in the recovery of TER in the ischemia-injured porcine ileum by measuring TER in ischemia-injured tissues in the presence of HOE-694 (25 μM). HOE-694 was applied to the apical (mucosal) surface at concentrations that inhibit NHE2 activity with no effect on NHE3 (33). Mucosal application of HOE-694 induced significant elevations (P < 0.01) in TER in ischemia-injured tissues (Fig. 3) that displayed similar kinetics to amiloride-treated tissues that were independent of changes in I_sc (data not shown). Furthermore, the effects of HOE-694 were not additive to those of PGE2, suggesting that HOE-694 and PGE2 are mediating their effects on TER via a common pathway. Overall, these data suggest that NHE2 is a critical target for PGE2 in the recovery of barrier function in the porcine ileum.

There are numerous studies (20, 36, 47) that have reported protective properties of NHE1 inhibitors in ischemia-reperfusion-injured porcine cardiac myocytes. In our intestinal ischemia model, inhibition of NHE2 appears to be responsible for the repair of ischemia-injured tissue. However, it is plausible that mucosal application of HOE-694, which is a potent inhibitor of NHE1, could equilibrate between mucosal and serosal surfaces, especially in the face of intestinal barrier dysfunction. To determine whether inhibition of NHE1 by HOE-694 was involved in the recovery of TER in the ischemia-injured ileum, we treated ischemia-injured tissues with HOE-694 (25 μM) on the serosal surface to inhibit the basolaterally expressed NHE1 isoform. Serosal HOE-694 had no effect on TER compared with nontreated ischemia-injured control tissue (Fig. 4). To further rule out NHE1 in the recovery of barrier function in the porcine ileum, we treated the serosal surface of ischemia-injured tissues with the selective NHE1 inhibitor HOE-642 (cariporide, 25 μM) which has been validated in the porcine species (47), but, as with serosal HOE-694, it was without effect with regard to TER.

**Transmucosal flux of Na⁺ in recovering ischemia-injured ileal mucosa in the presence of pharmacological inhibitors of NHE isoforms.** To confirm that the recovery of barrier function in the presence of HOE-694 was associated with inhibition of
NHE2-mediated Na\(^+\)/H\(^+\) exchange, bidirectional \(^{22}\text{Na}\) fluxes were performed in ischemia-injured mucosa in the presence of apical NHE isoform inhibitors. During the initial (30–60 min) and final (150–210 min) flux periods, net \(J_{\text{net}}\) rates from ischemia-injured control tissues treated with indomethacin were equal to \(\sim 1.9\) and \(1.7\, \mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}\), respectively, indicating a net absorptive state in the ischemia-injured mucosa (Table 1). Treatment of the ischemia-injured ileum with PGE\(_2\) or inhibitors of NHE activity reduced Na\(^+\) \(J_{\text{net}}\) compared with ischemia-injured control tissues. Reductions in Na\(^+\) \(J_{\text{net}}\) rates in the presence of these agents were attributable to reductions in Na\(^+\) \(J_{\text{ms}}\) rates rather than changes in Na\(^+\) rate \(J_{\text{ms}}\) (\(P < 0.05\)). In NHE1 inhibitory experiments, serosal (basolateral) application of HOE-694 resulted in elevated \(^{22}\text{Na}\) \(J_{\text{ms}}\) via reductions in \(J_{\text{net}}\) and \(J_{\text{net}}\) (\(J_{\text{ms}} = 5.8 \pm 1.0\), \(J_{\text{ms}} = 4.6 \pm 0.4\), \(J_{\text{net}} = 1.2 \pm 0.7\); \(P < 0.05\)). Calculated contributions of individual NHE isoforms to Na\(^+\) \(J_{\text{ms}}\) in ischemia-injured, indomethacin-treated tissues suggested that NHE2 and NHE3 contribute equally to electroneutral Na\(^+\) absorption during the initial and final flux periods (percentage of S-3226- and HOE-694-inhibited Na\(^+\) \(J_{\text{ms}}\) = 35 \pm 4.4\% and 38 \pm 4.6\%, respectively; Fig. 5). The percentage of Na\(^+\) \(J_{\text{ms}}\) inhibited by the nonselective NHE inhibitor amiloride was similar to that observed in PGE\(_2\)-treated tissues (percentage of amiloride and PGE\(_2\)-inhibited Na\(^+\) \(J_{\text{ms}}\) = 60.4 \pm 3.8\% and 56.3 \pm 2.3\%, respectively). Overall, these data demonstrate that both NHE2 and NHE3 contribute equally to brush-border electroneutral Na\(^+\) absorption in the ischemia-injured porcine ileum. However, significant reductions in tissue conductance were correlated with inhibition of NHE2 by HOE-694 but not with inhibition of NHE3 by S-3226.

**Recovery of TER in ischemia-injured mucosa treated with HOE-694 is independent of epithelial restitution.** Our previous studies have demonstrated that PG-mediated recovery of barrier function is mechanistically linked to modulation of the interepithelial tight junctions and paracellular space rather than enhanced rates of epithelial restitution in ischemia-injured villi (5, 16, 2). To confirm that inhibition of NHE2 by HOE-694 influenced TER via similar mechanisms, we performed histological and morphometric analyses on ischemia-injured tissues at selected time points during recovery. Immediately following ischemia, the ischemia-injured mucosa had denuded villus tips (Fig. 6B) that were near fully restituted within 60 min of mounting tissues on Ussing chambers (Figs. 6C and 7). These data demonstrate that elevations in TER observed in the presence of HOE-694 in this study, which occurred subsequent to evidence of restitution (i.e., after 60 min in vitro recovery), were independent of restitution. Furthermore, there were no noticeable histological or morphological differences between treatment groups, including PGE\(_2\) or inhibitors of HOE-694.

![Fig. 5. Quantitative contribution of NHE3 and NHE2 to mucosal-to-serosal Na\(^+\) fluxes in ischemia-injured porcine ileum. Results are expressed as percent control (ischemia-injured ileum treated with Indo alone) and represent means \(\pm\) SE; \(n = 5–6\) animals. Values represent combined \(^{22}\text{Na}\) fluxes measured over 2 consecutive 1-h flux periods (30–90 min and 90–150 min). NHE3 and NHE2-mediated Na\(^+\) fluxes were defined as mean mucosal-to-serosal flux of \(^{22}\text{Na}\) (\(J_{\text{ms}}\)) inhibited in the presence of mucosal S-3226 (20 \(\mu\text{M}\)) and HOE-694 (25 \(\mu\text{M}\)), respectively, in ischemia-injured ileum mounted on Ussing chambers. Combined NHE3- and NHE2-mediated Na\(^+\) \(J_{\text{ms}}\) were calculated as the percent reduction in Na\(^+\) \(J_{\text{ms}}\) in the presence of amiloride (total NHE). NHE3 and NHE2 contributed equally to Na\(^+\) \(J_{\text{ms}}\). The proportion of Na\(^+\) \(J_{\text{ms}}\) sensitive to application of PGE\(_2\) (1 \(\mu\text{M}\)) was similar to tissues treated with amiloride. \(^a\)Means within each flux period without a common superscript letter differ (\(P < 0.05\)).

### Table 1. Unidirectional Na\(^+\) fluxes across ischemia-injured porcine ileal mucosa treated with Indo, PGE\(_2\), or mucosal Na\(^+\)/H\(^+\) exchanger isoform inhibitors

<table>
<thead>
<tr>
<th></th>
<th>(J_{\text{ms}}) meq\cdot cm(^{-2})\cdot h(^{-1})</th>
<th>(J_{\text{ms}}) meq\cdot cm(^{-2})\cdot h(^{-1})</th>
<th>(J_{\text{ms}}) meq\cdot cm(^{-2})\cdot h(^{-1})</th>
<th>(G), mS/cm(^2)</th>
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<tr>
<td><strong>30–90 min flux period</strong></td>
<td></td>
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<tr>
<td>Indo</td>
<td>4.8 \pm 0.3(^a)</td>
<td>2.9 \pm 0.4(^a)</td>
<td>1.9 \pm 0.4(^a)</td>
<td>41.0 \pm 3.0(^a)</td>
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<tr>
<td>Indo/PGE(_2)</td>
<td>2.1 \pm 0.3(^b)</td>
<td>2.6 \pm 0.4(^b)</td>
<td>(-0.4 \pm 0.2(^b)</td>
<td>29.0 \pm 2.1(^b)</td>
</tr>
<tr>
<td>Indo/amiloride</td>
<td>1.9 \pm 0.6(^b)</td>
<td>2.7 \pm 0.7(^b)</td>
<td>(-0.9 \pm 0.6(^b)</td>
<td>36.2 \pm 4.1(^b)</td>
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<tr>
<td>Indo/HOE-694</td>
<td>3.1 \pm 0.5(^b)</td>
<td>3.3 \pm 0.4(^b)</td>
<td>(-0.1 \pm 0.2(^b)</td>
<td>35.3 \pm 3.0(^b)</td>
</tr>
<tr>
<td>Indo/S-3226</td>
<td>3.0 \pm 0.3(^b)</td>
<td>2.9 \pm 0.4(^b)</td>
<td>0.1 \pm 0.3(^b)</td>
<td>37.7 \pm 4.4(^b)</td>
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<tr>
<td><strong>150–210 min flux period</strong></td>
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<tr>
<td>Indo</td>
<td>4.1 \pm 0.1(^b)</td>
<td>2.4 \pm 0.4(^b)</td>
<td>1.7 \pm 0.6(^b)</td>
<td>32.0 \pm 4.0(^b)</td>
</tr>
<tr>
<td>Indo/PGE(_2)</td>
<td>2.0 \pm 0.4(^b)</td>
<td>1.6 \pm 0.8(^b)</td>
<td>(-1.0 \pm 0.5(^b)</td>
<td>21.0 \pm 2.0(^b)</td>
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<tr>
<td>Indo/amiloride</td>
<td>2.4 \pm 0.3(^b)</td>
<td>1.8 \pm 0.2(^b)</td>
<td>(-0.3 \pm 0.4(^b)</td>
<td>24.8 \pm 2.3(^b)</td>
</tr>
<tr>
<td>Indo/HOE-694</td>
<td>3.2 \pm 0.3(^b)</td>
<td>2.9 \pm 0.6(^b)</td>
<td>0.6 \pm 0.3(^b)</td>
<td>23.0 \pm 1.0(^b)</td>
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<tr>
<td>Indo/S-3226</td>
<td>3.2 \pm 0.6(^b)</td>
<td>2.7 \pm 0.5(^b)</td>
<td>0.5 \pm 0.1(^b)</td>
<td>33.8 \pm 3.7(^b)</td>
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Values are means \(\pm\) SE; \(n = 8\). Data are shown for fluxes performed during 30–90 and 150–210 min of the 240-min experiments shown in Figs. 3 and 4. Fluxes were commenced after treatment with either indomethacin (Indo; 5 \(\mu\text{M}\)) on both surfaces of the tissues and serosal 16,16-dimethyl PGE\(_2\) (PGE\(_2\); 1 \(\mu\text{M}\)), mucosal amiloride (1 \(\text{mM}\)), mucosal HOE-694 (25 \(\mu\text{M}\)), or mucosal S-3226 (25 \(\mu\text{M}\)) for successive 60-min periods. \(J_{\text{ms}}\), mucosal-to-serosal Na\(^+\) flux; \(J_{\text{ms}}\), serosal-to-mucosal Na\(^+\) flux; \(J_{\text{net}}\), net flux of Na\(^+\); \(G\), conductance. \(^a\)Means within a flux period without a common superscript letter differ (\(P < 0.05\)).

\[AJP-Gastrointest\ Liver Physiol \cdot VOL 291 \cdot NOVEMBER 2006 \cdot www.ajpgi.org\]
Immunolocalization of NHE2 and NHE3 in the porcine ileum. Recent studies (3, 10) have suggested differential localization patterns of NHE2 and NHE3 in the gastrointestinal tract. To determine if localization patterns of NHE2 and NHE3 may account for changes in barrier function, immunohistochemical analyses were performed on the control and ischemia-injured mucosa. These studies revealed that NHE3 and NHE2 are expressed predominantly in the villus epithelium, and this was unaltered by ischemic injury (Fig. 8).

Expression of NHE isoforms in the porcine ileum. Pharmacological inhibitor experiments revealed that NHE2 and NHE3 contribute equally to $^{22}\text{Na}^+$/H$^+$ flux across the ischemia-injured porcine ileum, but recovery of TER was linked with inhibition of NHE2. Therefore, we assessed the expression of NHE3 and NHE2 protein in the porcine ileum. Western blot analyses of ileal protein extracts revealed that both NHE2 and NHE3 are expressed as proteins of $\approx 85$ kDa in the porcine ileum (Fig. 9A). Furthermore, there was a significant increase in NHE2 and NHE3 protein levels in tissues immediately following ischemia compared with uninjured controls. Western blot analyses of ischemia-injured tissues treated with PGE2 (Fig. 9B) revealed downregulation of NHE2 protein expression under the influence of PGE2, whereas NHE3 expression was unchanged.

Coimmunoprecipitation of NHE2 with EBP50. It is well established that inhibition of apical NHE3 by PKA requires participation of Na$^+/H^+$ regulatory factors (NHERFs) bound to the cytoskeletal protein ezrin (45, 46). Whether similar cytoskeletal regulation exists with NHE2 has not been reported. To determine whether there was coexpression of NHE2 and NHERF proteins, we conducted coimmunoprecipitation experiments with NHE2 and NHERF1 (EBP50). As shown in Fig. 10, EBP50 was expressed in both NHE2 and NHE3 control immunoprecipitates. However, ischemic injury in-
protein expression was unchanged by PGE2 treatment. Anti-
is mediated by the ClC-2 Cl
initial rapid elevations in TER observed in the presence of PGs,
ent of this model, which appears to be responsible for the
response to application of PGE2 in recovering ischemia-injured mucosa. NHE3
periods on Ussing chambers. NHE2 protein expression was decreased in
injured mucosa by a mechanism involving regulation of two
Cl
channels expressed within the
brush-border NHE isoform in mammals. This
predominant brush-border NHE isoform in mammals.

DISCUSSION

PGs restore intestinal barrier function in the ischemia-
injured mucosa by a mechanism involving regulation of two
opposing ion transport pathways, 1) stimulation of electrogenic
Cl− secretion and 2) inhibition of electroneutral Na+ absorption. Recently, we
determined that the Cl− secretory component of this model, which appears to be responsible for the
initial rapid elevations in TER observed in the presence of PGs,
is mediated by the CIC-2 Cl− channels expressed within the
tight junctions (29). Elevations in TER observed during the
later stages of PG-mediated repair of the intestinal barrier
appear to be mediated by the inhibition of NHE-mediated Na+ absorption, as evidenced by the enhanced recovery of TER
observed in tissues treated with the apical NHE inhibitor
amiloride compared with ischemia-injured tissue treated with
indomethacin alone. Similar results were observed in an addi-
tional study in which NHE activity was abolished by substi-
tuting Na+ in the mucosal Ringer solution with D-glucamine
chloride. Taken together, these initial experiments confirmed
an important role for NHE inhibition in the reestablishment of
barrier function in the ischemia-injured porcine intestine.

To date, nine NHE isoforms have been identified in the
mammalian intestine (50). The major brush-border isoforms
contributing to electroneutral Na+/H+ exchange are NHE3 and
NHE2. We sought to determine whether NHE-dependant re-
covery of TER was due to inhibition of multiple isoforms or a
result of inhibition of a select isoform. NHE3 appears to be the
predominant brush-border NHE isoform in mammals. This
important role of NHE3 exchange is demonstrated in NHE3
knockout mice, which exhibit chronic diarrhea and distur-
bances in systemic fluid balance (22, 38). Therefore, we
initially investigated the effects of NHE3 inhibition with
S-3226 in the ischemia-injured ileum. However, application of
S3226 had no influence on TER, despite causing significant
reductions in Na+ Jm. Inhibition of NHE3 has been shown to
be involved in regulation of TER. Turner et al. (43) showed
that inhibition of NHE3 with S-3226 induced elevations in
TER that occurred only in the presence of activated Na+
-glucose cotransporter in Caco-2 cell monolayers. However, in
our Ussing chamber model, glucose is selectively added to the
serosal side of mucosal sheets to prevent activation of this
pathway, and thus the findings of Turner et al. may not be
entirely relevant to our observations in the ischemia-injured
mucosa. In addition, given the multitude of reports on the
protective effects of NHE1 inhibitors on ischemia-reperfusion
INHIBITION OF NHE2 MEDIATES BARRIER FUNCTION RECOVERY

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G892

AJP-Gastrointest Liver Physiol • VOL 291 • NOVEMBER 2006 • www.ajpgi.org

Injury in various tissues (20, 36, 47), we assessed the role of the basolaterally expressed NHE1 in the ischemia-injured ileum. However, blockade of NHE1 in the ischemia-injured mucosa with serosal application of the selective NHE1 inhibitors HOE-694 (25 μM) or HOE-642 (cariporide, 25 μM) had no effect on the recovery of TER, suggesting that NHE1 is not directly involved in mucosal barrier repair in this model.

The most important finding in these experiments was that inhibition of brush-border NHE2 with HOE-694 led to restoration of TER in the ischemia-injured ileum, indicating that NHE2 is intimately involved in the mucosal recovery process following ischemic injury. Until now, the physiological role of NHE2 in the gut has been unclear. Unlike NHE3 knockout mice, NHE2-null mice exhibit no overt disease phenotype and exhibit similar basal rates of net Na⁺ and Cl⁻ absorption compared with wild-type mice, suggesting a minimal contribution of this isoform to Na⁺/H⁺ exchange under basal conditions in this species (37). However, significant differences exist between species and the intestinal segment studied with regard to the quantitative contribution of NHE3 and NHE2 to Na⁺/H⁺ exchange. For example, in the canine ileum, Na⁺/H⁺ exchange is mediated solely by NHE3 (28), whereas in the avian colon, NHE2 is the major brush-border NHE (12). In the rabbit ileum, ~50% of basal Na⁺/H⁺ exchange occurs via NHE3 and 50% occurs via NHE2 (48). The quantitative contribution of NHE2 and NHE3 to basal electroneutral Na⁺/H⁺ exchange in the porcine ileum has not been directly studied. The present studies utilizing the ischemia-injured porcine ileum revealed that NHE2 and NHE3 contribute equally to electroneutral Na⁺ transport as demonstrated by HOE-694- and S-3226-inhibitable Na⁺ fluxes. Whether NHE3 and NHE2 contribute equally to intestinal brush-border Na⁺/H⁺ exchange under basal (nonischemic) conditions has yet to be determined. NHE2 activity and expression have been shown to be upregulated during metabolic acidosis, massive small bowel resection, and infection with enteropathogenic Escherichia coli infection, suggesting possible roles of this isoform during pathological states (19, 26, 30). NHE2 is also important for gastric parietal cell viability, as evidenced by parietal cell destruction and chronic gastritis in NHE2-null mice (7). More relevant to our studies, Nowak et al. (33) recently showed that the selective NHE1 inhibitor HOE-642, applied to the apical surface of Caco-2 monolayers at concentrations that also inhibit NHE2 (50 μM), prevented PMA-induced disruption of epithelial barrier function.

The mechanism by which targeted inhibition of NHE2 leads to recovery of barrier function is not well understood. Histological evaluation of recovering ischemia-injured mucosa indicates that elevations in TER under the influence of NHE2 blockade were independent of the migratory phase of epithelial restitution. Therefore, improvements in TER were likely due to decreased permeability of the paracellular space, as has been demonstrated in previous studies (5, 16, 24). NHE2 and NHE3 were both expressed in enterocytes of the apical villus of the porcine ileum and were both upregulated immediately following ischemia, suggesting that expression patterns do not explain the differential effects on TER between the two isoforms. One potential hypothesis for elevations in TER under the influence of PGE₂ or HOE-694 is the development of an osmotic gradient due to luminal accumulation of Na⁺ resulting from inhibition of Na⁺ absorption. Luminal osmotic loads have been shown to increase TER through a mechanism involving dehydration and collapse of the lateral intercellular space (5, 24, 27). Although conceivable, results from the present experiments do not entirely support this hypothesis. For example, pharmacological inhibition of NHE3 or NHE2 significantly reduced transmucosal Na⁺ fluxes and Jₑ/ₑ as well as TER in Caco-2 cells (47), suggesting that recovery of TER is selectively mediated through NHE2 regulation. Likewise, previous studies have shown that the PGE₂-evoked Cl⁻ secretory response that is critical for the initial elevations in TER is mediated through CIC-2 Cl⁻ channels, which represent only a small portion of PGE₂-stimulated Iₑ. Overall, these data imply that PGE₂-mediated recovery of TER in the ischemia-injured mucosa is not a result of changes in solute and osmotic fluid movement per se but a result of selective regulation of brush-border membrane proteins such as NHE2 and CIC-2. Regulation of such proteins may activate signaling cascades that result in closure of the tight junctions.

Results from the present studies suggest that restoration of TER is due to inhibition of NHE2 activity rather than changes in NHE2 expression. This is supported by the fact that the NHE2 inhibitor HOE-694 stimulated recovery of TER, a maneuver that would not be expected to acutely change NHE expression. However, it is interesting that PGE₂ induced a rapid downregulation of NHE2 expression but not NHE3. Transmucosal Na⁺ flux experiments suggest that PGE₂ inhibits both NHE2 and NHE3 in the ischemia-injured porcine ileum. The selective downregulation of NHE2 under the influence of PGE₂ may be due to inherent differences in the short-term regulation of NHE2 and NHE3. For example, NHE3 is regulated via recycling between the plasma membrane and endosomes, whereas NHE2 has a short half-life and undergoes lysosomal degradation (9, 23). This would essentially allow for more rapid regulation of NHE2 protein levels in the mucosa, which may explain the rapid changes in NHE2 protein levels in PGE₂-treated tissues in this study.

The mechanistic link between inhibition of NHE2 activity and repair of mucosal barrier function is unclear. However, NHE2-specific effects may be due to inherent differences in regulation of NHE2 compared with NHE3. Although NHE3 and NHE2 COOH termini have been shown to interact with the same regulatory proteins and contain both inhibitory and stimulatory domains (31), there exists considerable variation in their activity depending on the cell model or epithelium being studied. For example, NHE3 is inhibited by PKA and PKC, whereas NHE2 appears to be activated by these kinases in transfected PS120 fibroblasts (32). However, in C2bbe epithelial cells, both intracellular Ca²⁺ and cAMP inhibit NHE2 activity by changes in Vₑ max and KₑNa, whereas PKC has no effect on NHE2 activity (8).

It is well documented that inhibition of NHE3 activity by protein kinases requires the presence of NHERFs (46, 49, 51). These cytosolic scaffold molecules are a well-studied family of postsynaptic density-95/discs large/zonula occludens-1 (PDZ) domain-containing proteins, which consist of two orthologs, EBP50 (NHERF1) and E3KARP (NHERF2). The interaction and regulation of NHERF proteins have been characterized in a number of apical ion transporters, in particular NHE3 (21, 40). However, not much is known regarding NHERF interactions and regulation of other NHE isoforms, including NHE2.
Coimmunoprecipitation experiments in the present study revealed that EBPS0 is coexpressed with both NHE2 and NHE3 in the porcine ileal mucosa. Although NHE3-EBP50 PDZ interactions have been well documented, NHE2-EBP50 interactions have not been reported (50). An additional interesting finding in these experiments was the increased expression of EBP50 in NHE2 immunoprecipitates from ischemia-injured tissues. This interaction appears to be different than EBP50-NHE3 interactions in these tissues, which were unchanged in ischemic tissues. Our data are in agreement with studies (21) showing that EBP50 binds constitutively to NHE3 under basal and cAMP-stimulated conditions. With regard to NHE2-EBP50 interactions observed in this study, NHERF protein-protein interactions have been shown to be highly regulated under stimulated conditions. For example, stimulation of the β2-adrenergic receptor with β2-agonists results in rapid recruitment and binding of EBP50 to the COOH terminus of the β2-adrenergic receptor (17). The association of EBP50 with NHE2 and its relevance to intestinal barrier repair are unclear at this point. Furthermore, it is not known whether EBP50 expression in NHE2 immunoprecipitates represents a direct association of EBP50 with NHE2 or is part of a larger complex of proteins associated with NHE2. Further studies specifically focusing on NHE2-EBP50 interactions and their relevance to recovery of paracellular permeability are needed.

GRANTS

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-53284 and United States Department of Agriculture National Research Initiative 2003-35204-13231.

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AJP-Gastrointest Liver Physiol • VOL 291 • NOVEMBER 2006 • www.ajpgi.org


