Recovery of mucosal barrier function in ischemic porcine ileum and colon is stimulated by a novel agonist of the ClC-2 chloride channel, lubiprostone

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Moeser AJ, Nighot PK, Engelke KJ, Ueno R, Blikslager AT. Recovery of mucosal barrier function in ischemic porcine ileum and colon is stimulated by a novel agonist of the ClC-2 chloride channel, lubiprostone. Am J Physiol Gastrointest Liver Physiol 292: G647–G656, 2007. First published October 19, 2006; doi:10.1152/ajpgi.00183.2006.—Previous studies utilizing an ex vivo porcine model of intestinal ischemic injury demonstrated that prostaglandin (PG)E2 stimulates repair of mucosal barrier function via a mechanism involving Cl– secretion and reductions in paracellular permeability. Further experiments revealed that the signaling mechanism for PGE2-induced mucosal recovery was mediated via type-2 Cl– channels (ClC-2). Therefore, the objective of the present study was to directly investigate the role of ClC-2 in mucosal repair by evaluating mucosal recovery in ischemia-injured intestinal mucosa treated with the selective ClC-2 agonist lubiprostone. Ischemia-injured porcine ileum was mounted in Ussing chambers, and short-circuit current (Isc) and transepithelial electrical resistance (TER) were measured in response to lubiprostone. Application of 0.01–1 μM lubiprostone to ischemia-injured mucosa induced concentration-dependent increases in TER, with 1 μM lubiprostone stimulating a twofold increase in TER (ΔTER = 26 Ω·cm2; P < 0.01). However, lubiprostone (1 μM) stimulated higher elevations in TER despite lower Isc responses compared with the nonselective secretory agonist PGE2 (1 μM). Furthermore, lubiprostone significantly (P < 0.05) reduced mucosal-to-serosal fluxes of 3H-labeled mannitol to levels comparable to those of normal control tissues and restored occludin localization to tight junctions. Activation of ClC-2 with the selective agonist lubiprostone stimulated elevations in TER and reductions in mannitol flux in ischemia-injured intestine associated with structural changes in tight junctions. Prostanes such as lubiprostone may provide a selective and novel pharmacological mechanism of accelerating recovery of acutely injured intestine compared with the nonselective action of prostaglandins such as PGE2.

ischemia; type 2 chloride channels; repair

THE INTESTINAL BARRIER is composed of a single layer of columnar epithelium that serves as the body’s first line of defense against a hostile environment within the intestinal lumen (18, 21, 35). Barrier properties of the epithelium are in large part regulated by interepithelial tight junctions (TJs), which reside at the apical-most region of the paracellular space (3, 34, 36). TJs polarize the cell into apical and basolateral regions (fence function) and regulate passive diffusion of solutes and macromolecules (gate function) (3).

Intestinal ischemia is an important mechanism of intestinal barrier injury (27, 31, 40). Ischemic injury causes disruption of the TJ protein complexes and enhances epithelial permeability, permitting transmigration of luminal bacterial toxins and antigens into subepithelial tissues and the circulation (44). Such mucosal injury has resulted in high mortality rates ranging between 59% and 93% (2, 28, 44). It is also becoming increasingly evident that many critically ill patients suffer from multiple organ failure initiated by poor splanchnic perfusion and resultant loss of intestinal barrier properties (17, 31, 41). Multiple organ failure is the leading cause of death in intensive care unit patients (31).

The molecular mechanisms of ischemia-induced disruption of barrier function have been studied predominantly in cell models that mimic cellular events that occur during ischemia, such as ATP depletion/repletion and Ca2+ switch assays (14, 47, 55). These models demonstrate that the critical event defining disruption of barrier function is the loss of TJ architecture and redistribution of TJ proteins such as occludin and zonula occludens-1 (ZO-1) from the apical TJs. TJ reparative events involve recruitment and reorganization of occludin and ZO-1 to the apical tight junctional region. However, these mechanisms are poorly understood.

In our previous work, we demonstrated a critical role for Cl– secretion in the repair of intestinal barrier function in ischemia-injured porcine ileum. Activation of Cl– secretory pathways with the nonselective secretory agonist prostaglandin (PG)E2 triggered rapid recovery of transepithelial electrical resistance (TER) in ischemia-injured ileum and reduced mucosal-to-serosal fluxes of 3H-labeled mannitol (9, 11, 12, 37). Inhibition of basolateral Cl─ uptake in these tissues abolished the PGE2-mediated secretory response and prevented full restoration of TER levels, confirming the important role of Cl– secretion in mucosal barrier repair in this model (11, 37). Recent studies utilizing selective Cl– channel inhibitors revealed that recovery of TER is mediated solely through type 2 chloride channels (CIC-2) expressed in the apical TJ of restituted villus epithelium (37).

The CIC-2 Cl– channel is expressed in a variety of mammalian secretory epithelia and epithelial cell lines but plays a relatively minor role in Cl– transport and fluid secretion (8, 38, 46). Knockout of the CIC-2 gene disrupts normal development of retinal pigment epithelium and the blood-testis barrier (13), although there were no measurable effects on basal secretion [in terms of short-circuit current (Isc)] in CIC-2-null mouse colonic epithelia (56). There is also increasing evidence for a role of CIC-2 in absorptive processes because of its basolateral localization in duodenal and colonic surface epithelium (15, 42) CIC-2 has also been localized to the interepithelial TJ.

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colocalized with TJ proteins ZO-1 and occludin in mouse and porcine intestinal mucosa (23, 29, 37). Activation of CIC-2 occurs under various physiological conditions and in periods of cellular stress (1, 8, 24, 43, 45). The exact role of CIC-2 in intestinal injury and repair is poorly understood.

Lubiprostone (Sucampo Pharmaceuticals) is a novel bicyclic fatty acid of the prostone group that has demonstrated selectivity for activation of CIC-2 Cl⁻ currents in human colonic T84 and CIC-2-transfected human embryonic kidney (HEK) cells (16). Lubiprostone has been shown to induce intestinal fluid secretion when administered orally (53) and has demonstrated clinical efficacy in treatment of patients with constipation (25, 26). The objective of the present experiment was to evaluate the effects of lubiprostone on restoration of mucosal barrier function in the ischemia-injured porcine intestine.

METHODS

Experimental porcine surgeries. All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Six- to 8-wk-old Yorkshire crossbred pigs of either sex were housed individually and maintained on a commercial pelleted feed. Pigs were held off feed for 24 h before 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 intermittent infusion of thiopental (6–8 mg·kg⁻¹·h⁻¹). Pigs were placed on a heating pad and ventilated with 100% O₂ via a tracheotomy with a time-cycled ventilator. The jugular vein and carotid arteries were cannulated, and blood gas analysis was performed to confirm normal pH and partial pressures of CO₂ and O₂. Lactated Ringer solution was administered intravenously at a maintenance rate of 15 ml·kg⁻¹·h⁻¹. The ileum and colon were approached via a ventral midline incision. Ileal or colonic segments were delineated by ligating the intestine at 10-cm intervals and subjected to ischemia by occluding the local mesenteric blood supply for 45 min. For colonic studies, the midregion of the ascending colon was selected.

Ussing chamber studies. After the 45-min ischemic period, tissues were harvested from the pig and the mucosa was stripped from the seromuscular layer in oxygenated (95% O₂: 5% CO₂) Ringer solution (mM: 154 Na⁺, 6.3 K⁺, 137 Cl⁻, 0.3 H₂PO₄, 1.2 Ca²⁺, 0.7 Mg²⁺, 24 HCO₃⁻, pH 7.4) containing 5 μM indomethacin to prevent endogenous PG production during the stripping procedure. Tissues were then mounted in 3.14-cm²-aperture Ussing chambers, as described in previous studies (4). For Ussing chamber experiments, ileal or colonic tissues from one pig were mounted on multiple Ussing chambers and subjected to different in vitro treatments. Mean data are representative of six Ussing chamber experiments (n = 6 animals). Tissues were bathed on the serosal and mucosal sides with 10 ml of Ringer solution. The serosal bathing solution contained 10 mM glucose and was circulated on a chamber unit area basis. TER (Ω·cm²) was calculated from the spontaneous PD and 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 tissues were mounted on Ussing chambers, they were allowed to acclimate for a period of 30 min to achieve stable baseline measurements, after which experimental treatments were added. Lubiprostone (−)-7-[(2R,4aR,5R,7aR)-2-(1,1-difluoropropyl)-2-hydroxy-6-oxooctahydropyrido[4,3-b]pyran-5-yl]heptanoic acid) was added to the mucosal side of tissues, except in select experiments that assessed the effects of serosal treatment with lubiprostone. PGE₂ was added to the serosal side of tissues to stimulate epithelial repair. In studies investigating the Cl⁻ channel selectivity of lubiprostone, tissues were pretreated (t = 0 min) with pharmacological Cl⁻ channel inhibitors on the appropriate surface and then treated with lubiprostone (1 μM) on the serosal mucosal side of the tissue (t = 30 min).

Mucosal-to-serosal fluxes of [³H]mannitol. To assess mucosal permeability after experimental treatments, 0.2 μCi/ml [³H]mannitol was placed on the mucosal side of Ussing chamber-mounted tissues. After a 15-min equilibration period, standards were taken from the mucosal side of each chamber and a 60-min flux period was established by taking 0.5-ml samples from the serosal compartment. The presence of [³H] was established by measuring β-emission in a liquid scintillation counter (LKB Wallac, model 1219 Rack Beta, Perkin Elmer Life and Analytical Sciences, Boston, MA). Unidirectional [³H]mannitol fluxes from mucosa to serosa were evaluated by determining mannitol-specific activity added to the mucosal bathing solution and calculating the net appearance of tritium over time in the serosal bathing solution on a chamber unit area basis.

Histological examination. Tissues were taken at 0, 60, and 240 min for routine histological evaluation. Tissues were sectioned (5 μm) and stained with hematoxylin and eosin. For each tissue, three sections were evaluated. For ileal tissues, four well-oriented villi and crypts were identified in each section. Villus length was obtained with a micrometer in the eyepiece 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 following formula: villus surface area = 2πr⁰1/2[(4/π)r]dh, where d is villus diameter (width) at midpoint and h is villus height.

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 (5). 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.

Immunofluorescence labeling of occludin. Immunofluorescence labeling was performed on ileal tissues that were embedded in optimal cutting temperature medium, frozen, sectioned at 5-μm thickness, and fixed in cold 2-methylbutane (Sigma-Aldrich). Tissue sections were blocked with 2% BSA before incubation with rabbit anti-occludin polyclonal antibody (1:150, Zymed, San Francisco, CA) in normal rabbit serum for 2 h at 4°C. Sections were washed with PBS and incubated for 45 min with FITC-conjugated anti-rabbit secondary antibody (1:50; Zymed) in the dark. Sections were mounted, and well-oriented villi were examined with a Vanox AHS-3 Photomicroscope linked to a Spot RT Slider cooled charge-coupled device digital camera.

Chemicals. Indomethacin, 16,16-dimethyl-PGE₂, ZnCl₂, bumetanide, and [³H]mannitol were purchased from Sigma (St. Louis, MO). N-(4-methylphenylsulfonyl)-N'(4-trifluoromethylphenyl)urea (DASU-02) was generously provided by B. D. Shultz (Kansas State University, Manhattan, KS). Lubiprostone was obtained from Sucampo Pharmaceuticals.

Statistical analysis. Data are reported as means ± SE. All data were analyzed by using an analysis of variance (ANOVA) for repeated measures, except where the peak response was analyzed by using a standard one-way ANOVA (SigmaStat, Jandel Scientific, San Rafael, CA). Tukey’s procedure for multiple comparisons was used to determine pairwise differences between treatments.

RESULTS

Effect of the CIC-2 agonist lubiprostone on Isc and TER in ischemia-injured porcine ileum. Porcine ileum was subjected to 45 min of mesenteric ischemia and mounted on Ussing chambers for measurement of TER and Isc over a 180-min
recovery period. Tissues subjected to ischemia had significantly lower starting TER values (by ~40%) compared with nonischemic control tissue, indicating significant disruption of intestinal barrier function in these tissues. Application of the nonselective secretory agonist PGE₂ (1 μM) to the serosal side of ischemic tissues induced elevations in TER (ΔTER = 26 Ω·cm²) that attained preischemic control levels within 45 min of treatment with PGE₂. Application of the ClC-2 agonist lubiprostone (0.1 and 1 μM) to the mucosal side of ischemia-injured mucosa induced concentration-dependent increases in TER (Fig. 1A), with 1 μM lubiprostone stimulating an approximately twofold increase in TER (ΔTER = 25 Ω·cm²; P < 0.01). Concentration-dependent increases in Iₑ were observed in response to increasing concentrations of lubiprostone (Fig. 1, B and C). Linear regression analysis revealed a significant correlation (r = 0.67, P = 0.01) between the magnitude of the Iₑ response induced by 1 μM lubiprostone (in terms of ΔIₑ) and the TER recovery response (in terms of ΔTER), whereas no such correlation existed in ischemic tissue treated with PGE₂ (r = 0.06, P = 0.82) (Fig. 2). The significant correlation between lubiprostone-stimulated Iₑ and TER may represent a more selective nature on ClC-2 Cl⁻ channel activity compared with PGE₂. However, it is unclear why no correlation existed with PGE₂ treatment, as this agent would presumably have a graded effect on Cl⁻ transport mediated via ClC-2 even if it also had effects on other Cl⁻ channels.

Effect of lubiprostone on mucosal-to-serosal flux of mannitol in ischemia-injured porcine ileum. As an alternative assessment of barrier function, mucosal-to-serosal fluxes of [³H]mannitol were examined on ischemia-injured tissues mounted in Ussing chambers in the presence of mucosal lubiprostone (1 μM). In line with TER responses, ischemia-

Fig. 1. Electrical responses of ischemia-injured porcine ileal mucosa to treatment with the selective type 2 Cl⁻ channel (ClC-2) agonist lubiprostone. Values represent means ± SE; n = 6. A: application of lubiprostone (0.1 and 1 μM doses) to the mucosal side of ischemic tissues bathed in indomethacin (Indo, 5 μM) induced marked elevations in transepithelial electrical resistance (TER) when added after a 30-min equilibration period (arrow). Application of prostaglandin (PG)E₂ (1 μM) to the serosal side of ischemia-injured mucosa induced significant elevations in TER that were lesser in magnitude than those from lubiprostone treatment. Statisticl analysis of TER data included a 2-way ANOVA on repeated measures. B: PGF2 and 1 μM lubiprostone induced rapid elevations in short-circuit current (Iₑ) that peaked at 15 min after treatment and gradually returned to basal levels over the 180-min recovery period. C: a significant (P < 0.05) increase in the absolute change in Iₑ (ΔIₑ) was observed in response to treatment with increasing concentrations of lubiprostone. ΔIₑ values represent the absolute change in Iₑ attained over a 30-min period following lubiprostone treatment. Data were analyzed with a 1-way ANOVA. Significant differences: *P < 0.05 vs. indomethacin; †P < 0.05 vs. all other treatments; ††P < 0.05 vs. all other treatments.

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injured tissues exhibited increased flux of $[^{3}H]$mannitol compared with noninjured control tissue ($P < 0.05$, 1-way ANOVA; Fig. 3), whereas ischemia-injured tissues treated with $1 \mu M$ lubiprostone had significantly reduced mannitol fluxes compared with nontreated ischemic tissues ($P < 0.05$) that were similar to those of control tissues.

**Effects of serosal application of lubiprostone to ischemic mucosa.** Because lubiprostone is an agonist of apical ClC-2 Cl$^{-}$ secretion, lubiprostone was applied to the mucosal side of tissues in these initial experiments. However, classic secretory agonists (such as PGE2) generally exert their effects on intestinal epithelium by binding to basolateral receptors, inducing signaling cascades ultimately leading to activation of apical Cl$^{-}$ transport and fluid movement (19). To determine whether the action of lubiprostone is preferential to the mucosal side of tissue, ischemia-injured tissues were treated with lubiprostone on either the mucosal or the serosal surface and both TER and $I_{sc}$ were measured. As demonstrated in earlier experiments, mucosal application of lubiprostone ($1 \mu M$) to ischemia-injured mucosa stimulated significant elevations in $I_{sc}$ ($\Delta I_{sc} = 28 \pm 4 \mu A/cm^2$) and TER ($\Delta$TER = $24 \pm 2 \Omega \cdot cm^2$) (Fig. 4). Serosal lubiprostone treatment induced minor but statistically significant elevations in $I_{sc}$ ($\Delta I_{sc} = 7 \pm 1.2 \mu A/cm^2$) compared with nontreated ischemic tissues ($P < 0.05$). However, serosal lubiprostone $I_{sc}$ responses were sig-

**Fig. 2.** Correlation between $\Delta I_{sc}$ and $\Delta$TER in ischemia-injured tissues treated with indomethacin ($5 \mu M$) and lubiprostone ($1 \mu M$) or indomethacin and PGE$_2$ ($1 \mu M$). There was a significant correlation between the magnitude of the $I_{sc}$ response ($\Delta I_{sc}$) induced by lubiprostone and the absolute change in TER ($\Delta$TER) in ischemia-injured tissues. In ischemia-injured tissues treated with PGE$_2$, $\Delta I_{sc}$ was poorly correlated with $\Delta$TER. The correlation coefficient ($r$) and its significance ($P$ values) are indicated adjacent to respective linear regression lines.

**Fig. 3.** Mucosal-to-serosal fluxes of $[^{3}H]$labeled mannitol across tissues treated with indomethacin ($5 \mu M$) or indomethacin and lubiprostone ($1 \mu M$). A single 60-min flux period was initiated after a 30-min equilibration period following addition of treatments. Ischemia-injured tissues bathed in indomethacin ($5 \mu M$) demonstrated marked elevations in TER in the presence of mucosal lubiprostone ($1 \mu M$), added after a 30-min equilibration period (arrow). Lubiprostone applied to the serosal side of ischemic mucosa had no influence on TER values. The $I_{sc}$ response in tissues treated with serosal lubiprostone was lesser in magnitude compared with mucosal lubiprostone. $\Delta I_{sc}$ values represent the absolute change in $I_{sc}$ attained over a 30-min period following lubiprostone treatment. $\Delta I_{sc}$ data were analyzed with a 1-way ANOVA. Significant differences: *$P < 0.01$ vs. indomethacin; *$P < 0.05$ vs. both indomethacin and lubiprostone.

**Fig. 4.** Effect of serosal vs. mucosal application of lubiprostone in ischemia-injured porcine ileum. Values represent means$\pm$SE; $n = 6$. A: ischemia-injured tissues bathed in indomethacin ($5 \mu M$) demonstrated marked elevations in TER in the presence of mucosal lubiprostone ($1 \mu M$), added after a 30-min equilibration period (arrow). Lubiprostone applied to the serosal side of ischemic mucosa had no influence on TER values. Statistical analysis of TER data included a 2-way ANOVA on repeated measures $B$: a significant ($P < 0.05$) increase in $I_{sc}$ was observed in response to mucosal treatment with lubiprostone. The $I_{sc}$ response in tissues treated with serosal lubiprostone was lesser in magnitude compared with mucosal lubiprostone. $\Delta I_{sc}$ values represent the absolute change in $I_{sc}$ attained over a 30-min period following lubiprostone treatment. $\Delta I_{sc}$ data were analyzed with a 1-way ANOVA. Significant differences: *$P < 0.01$ vs. indomethacin; *$P < 0.05$ vs. both indomethacin and lubiprostone.
significantly lower compared with mucosal lubiprostone treatment \((P < 0.01)\) and failed to evoke elevations in TER compared with ischemia-injured control tissues. The biological importance of this serosal response is unclear. It is conceivable that the serosal \(I_{sc}\) responses to lubiprostone could be due to translocation of this agent across leaky, injured epithelium. To test this, additional experiments were performed in which lubiprostone was applied to the mucosal and serosal surfaces of uninjured control tissues. Mucosal and serosal application of lubiprostone induced significant \(I_{sc}\) responses \((P < 0.05)\) in control tissues. However, as with ischemic tissues, a significantly attenuated \(I_{sc}\) response was observed with serosal application of lubiprostone compared with mucosal application (by 3.4-fold) in these tissues \((\Delta I_{sc} = 48.2 \pm 14.3 \text{ and } 14.2 \pm 5.3 \mu A/cm^2)\) in mucosally treated and serosally treated control tissues, respectively; \(P < 0.05\). Overall, this suggests that lubiprostone exhibits more activity on mucosal surfaces of porcine intestinal tissues regardless of whether the tissues are injured. Whether serosal \(I_{sc}\) responses by lubiprostone are due to activation of non-CIC-2 pathways requires further investigation.

*Histological evaluation of recovering ischemia-injured mucosa treated with lubiprostone.* To determine whether improvements in barrier function in response to lubiprostone treatment were due to enhanced epithelial restitution rates, histological and morphological analyses of epithelial restitution on recovering ischemic tissues mounted on Ussing chambers, the intestinal villi had undergone rapid and complete restitution as evidenced by contracted villi and migrating epithelial cells that covered the surface of injured villi (Fig. 5C). By the end of the 180-min recovery period, the restituted epithelial cells regained their normal columnar appearance (Fig. 5D), which was indistinguishable from normal control tissue (Fig. 5A). Overall, these data show that recovery of mucosal barrier function in the presence of lubiprostone was independent of the rate of epithelial restitution.

**Role of Cl\(^-\) secretion on lubiprostone-induced recovery of TER.** To gain further insight into the role of Cl\(^-\) secretion in lubiprostone-induced recovery of TER, ischemia-injured ileal mucosa was pretreated with the basolateral Na\(^+\)-K\(^+\)–2Cl\(^-\) cotransporter (NKCC1) inhibitor bumetanide (400 \(\mu M\)), and TER and \(I_{sc}\) were recorded in response to treatment with lubiprostone (1 \(\mu M\)). As shown in Fig. 6, pretreatment of ischemia-injured mucosa with bumetanide abolished the lubiprostone-induced \(I_{sc}\) and significantly inhibited initial rapid elevations in TER (Fig. 6).

Lubiprostone was shown previously to selectively activate CIC-2 with no effect on the cystic fibrosis transmembrane conductance regulator (CFTR) in transfected HEK cells (16). To determine whether the mucosal barrier reparative properties of lubiprostone were linked to targeted CIC-2 channel activation in our model, ischemic mucosa were pretreated with pharmacological inhibitors of CIC-2 and CFTR and TER and \(I_{sc}\) were measured in response to mucosal addition of lubiprostone (1 \(\mu M\)). Pretreatment of ischemic mucosa with the CIC-2 inhibitor ZnCl\(_2\) (300 \(\mu M\)) inhibited but did not abolish lubiprostone-induced \(I_{sc}\) (Fig. 7B). This correlated with impaired recovery of TER (expressed as % increase in TER; Fig. 7A). On the other hand, the CFTR inhibitor DSU-02 (300 \(\mu M\)) had no effect on \(I_{sc}\) or recovery of TER. Why lubiprostone-evoked \(I_{sc}\) was only partially sensitive to ZnCl\(_2\) while abolishing recovery of TER in these tissues is unclear. This response may be due to nonspecific secretory pathways not involved in repair of barrier function.

**Occludin immunofluorescence in ischemia-injured mucosa treated with lubiprostone.** Occludin is an integral membrane protein expressed exclusively in the interepithelial TJJs (49 – 52). The recruitment of occludin to the apical intercellular region represents the final stage of TJ formation or restoration (3a, 5a, 43a). Therefore, we performed immunofluorescence analyses of occludin to determine whether lubiprostone-stimulated recovery of barrier function in ischemic tissues was dependent on occludin relocalization (Fig. 7C). Quantification of occludin intensity from immunofluorescent images showed recovery of occludin expression at 180 min of recovery (Fig. 7D). This was not observed in tissues treated with lubiprostone alone (Fig. 7E). As with TER, the recovery of occludin expression was abolished by pretreatment with ZnCl\(_2\) (Fig. 7F). It is possible that recovery of TER and occludin expression are dependent on similar, yet distinct, cellular mechanisms.

<table>
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<th>Treatment</th>
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</tr>
<tr>
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<td>6.6±2.6*</td>
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<tr>
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<td>60</td>
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Data represent means ± SE; \(n = 6\). Immediately after ischemia, ~30% of the surface area of the villus is denuded as a result of epithelial sloughing. The denuded tips of villi were progressively restituted despite the presence of indomethacin (Indo), and the % surface area of the villus that is denuded was significantly less at 60 min than at the preceding time points \((P < 0.05, 1\text{-way ANOVA})\). However, treatment with lubiprostone (1 \(\mu M\)) had no further effect on denuded villous surface area at 60 min compared with treatment with Indo alone.

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**Fig. 5.** Histological appearance of ischemia-injured porcine ileal mucosa. **A:** uninjured control mucosa. **B:** ischemic injury resulted in lifting and sloughing of epithelium from the tips of villi when assessed immediately after ischemia. **C:** after a 60-min recovery period in an Ussing chamber in the presence of indomethacin (5 \(\mu M\)), villi have contracted and epithelial restitution is nearly complete. **D:** ischemia-injured tissue measured at the end (180 min) of the Ussing chamber recovery period. Bar = 100 \(\mu m\).
tissues was associated with restoration of tight junction structure. In control (nonischemic) tissues, occludin was localized to the apical intercellular junction region of ileal mucosa (Fig. 8A). However, in ischemia-injured tissue, occludin staining patterns were diffuse, with predominant intracellular localization (Fig. 8B). In ischemia-injured mucosa treated with lubiprostone (1 μM), occludin was localized predominantly to the apical TJs, similar to control tissues. Ischemic tissues that were pretreated with the ClC-2 blocker ZnCl₂ (300 μM, mucosal side) and then exposed to lubiprostone exhibited disorganized staining patterns of occludin similar to untreated ischemic tissues. Overall, these results suggest that a potential mechanism by which lubiprostone restores barrier function involves shifting of occludin from the cytosol to the lateral intercellular membrane and TJs.

Iₜₑ and TER in response to lubiprostone in ischemic porcine colon. To test whether lubiprostone-mediated repair mechanisms in ischemic intestine occurred in other regions of the intestinal tract, studies with lubiprostone in ischemia-injured porcine colon were performed. As in studies on the porcine ileum, the midregion of the porcine ascending colon subjected to 45 min of ischemia exhibited significant reductions in starting TER values compared with uninjured control tissue (TER = 117 ± 1.7 Ω·cm² in uninjured control vs. 25 ± 2.3 Ω·cm² in ischemic colonic mucosa; P < 0.01). Application of mucosal lubiprostone (1 μM) to ischemia-injured colon stimulated elevations in TER that were similar in magnitude to serosal PGE₂ (1 μM) treatment (Fig. 9A). Elevations in TER were linked with reductions in [³H]mannitol flux as observed in the ileum (Fig. 9B). Compared with the ileum, lubiprostone

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G652 C.L.C-2 MEDIATES RECOVERY OF BARRIER FUNCTION

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Fig. 6. Electrical responses in ischemia-injured porcine ileum in response to lubiprostone and the Na⁺-K⁺-2Cl⁻ cotransporter (NKCC1) inhibitor bumetanide. All data points represent means ± SE; n = 6. A: pretreatment of ischemia-injured porcine mucosa with indomethacin (5 μM) and with the basolateral NKCC1 inhibitor bumetanide (400 μM) significantly inhibited lubiprostone-induced elevations in TER (P < 0.05; ANOVA on repeated measures). B: treatment of ischemia-injured mucosa with bumetanide abolished lubiprostone-stimulated Iₑ. Lubiprostone was given at 1 μM. Lubiprostone significantly increased Iₑ compared with indomethacin alone (⁎P < 0.01). ΔIₑ values represent the absolute change in Iₑ attained over a 30-min period following lubiprostone treatment.

Fig. 7. Electrical responses of ischemia-injured porcine ileal mucosa to treatment with lubiprostone and Cl⁻ channel inhibitors. Values represent means ± SE; n = 6. A: ischemia-injured tissues bathed in indomethacin (5 μM) and treated with lubiprostone (1 μM) exhibited increases in TER (indicated by % increase in TER) compared with ischemia-injured tissues treated with indomethacin alone (⁎P < 0.05). Pretreatment with ZnCl₂ (300 μM) inhibited lubiprostone-induced elevations in TER (P < 0.05), whereas N-(4-methylphenylsulfonfonyl)-N′-(4-trifluoromethylphenyl)urea (DASU-02; 300 μM) was without effect on lubiprostone-induced elevations in TER (P > 0.05). B: a significant (P < 0.05) increase in the absolute change in Iₑ (ΔIₑ) was observed in response to treatment with lubiprostone. Application of ZnCl₂ significantly inhibited lubiprostone-stimulated Iₑ (P < 0.05), whereas pretreatment of tissues with DASU-02 had no effect. ΔIₑ values represent the absolute change in Iₑ attained over a 60-min period following lubiprostone treatment. Data were analyzed with a 1-way ANOVA. Significant differences: *P < 0.5 vs. indomethacin, *P < 0.05 vs. all other treatments.
elicited minor but significant ($P < 0.05$) elevations in $\text{I}_\text{sc}$ ($\Delta \text{I}_\text{sc} = 10 \pm 1.0 \ \mu \text{A/cm}^2$) in colonic epithelium compared with non-treated ischemia-injured controls ($\Delta \text{I}_\text{sc} = 3 \pm 0.9 \ \mu \text{A/cm}^2$). Application of PGE2 resulted in a trend ($P = 0.08$, 1-way ANOVA, Tukey’s test) for increased $\text{I}_\text{sc}$ in ischemic tissues ($\Delta \text{I}_\text{sc} = 6 \pm 0.8 \ \mu \text{A/cm}^2$) (Fig. 9C).

Histological analysis revealed that the ischemia caused denudation of the surface epithelium (Fig. 10D). Within 60 min of mounting tissues on Ussing chambers, the epithelial monolayer was restored and composed of predominantly flattened epithelial cells that had migrated from the crypts onto the

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**Fig. 8.** Occludin immunofluorescence in ischemia-injured tissues treated with lubiprostone and ZnCl2. A: control, uninjured ileal mucosa after 180 min on Ussing chambers. Note the staining of occludin at the apical intercellular tight junctions (indicated by arrows). B: ischemia-injured mucosa after a 180-min in vitro recovery period in the presence of indomethacin (5 μM). Note the disorganized appearance of occludin fluorescence, with a lack of accumulation of occludin at the region of the interepithelial junctions. C: occludin fluorescence patterns in lubiprostone-treated ischemic mucosa (1 μM lubiprostone) were predominantly localized at the lateral intercellular space and tight junction (arrows). D: ischemia-injured mucosa treated with lubiprostone (1 μM) and ZnCl2 (300 μM, mucosal surface) after a 180-min in vitro recovery period in the presence of indomethacin (5 μM). Note the disorganized appearance of occludin fluorescence as seen in untreated tissues (A).

**Fig. 9.** Electrical responses of ischemia-injured porcine colon to treatment with the selective CLC-2 agonist lubiprostone. Bars represent means ± SE; $n = 5–6$ animals. A: porcine ischemia-injured colon bathed in indomethacin (5 μM) demonstrated marked elevations in TER in the presence of lubiprostone (1 μM), added after a 30-min equilibration period (arrow). Statistical analysis of TER data included a 2-way ANOVA on repeated measures. B: ischemia-injured colonic tissues in the presence of indomethacin alone were significantly more permeable to mannitol compared with control tissues, whereas ischemia-injured tissues treated with 1 μM lubiprostone had significantly reduced mannitol fluxes that were similar to those of control tissues. C: a significant ($^*P < 0.05$) increase in $\Delta \text{I}_\text{sc}$ was observed in response to treatment with lubiprostone. $\Delta \text{I}_\text{sc}$ values represent the absolute change in $\text{I}_\text{sc}$ attained over a 60-min period following lubiprostone treatment. $\Delta \text{I}_\text{sc}$ and [3H]mannitol flux data were analyzed with a 1-way ANOVA; significant differences were observed ($P < 0.05$).
surface of the basal lamina, as seen in Fig. 10D. By the end of the 180-min recovery period, the surface epithelium of all ischemia-injured tissues had taken on a columnar appearance, irrespective of treatment (Fig. 10D), that was similar to normal control tissue (Fig. 10A).

**DISCUSSION**

Mechanisms responsible for restoration of mucosal barrier function in acutely injured intestinal mucosa include two major events: epithelial restitution and closure of the paracellular space and TJs. Epithelial restitution is the initial reparative event that involves villous contraction and epithelial cell migration, which act in concert to rapidly restore epithelial continuity, a process that is independent of cellular proliferation (10, 11, 22, 39). Closure of the paracellular space involves reassembly of the interepithelial TJ protein complexes and restoration of epithelial barrier function (6, 47, 48, 54).

We have accumulated evidence for a critical role of ClC-2 mediated Cl\(^{-}\) secretion in recovery of mucosal barrier function in ischemia-injured intestine (37). The objective of the present studies was to test the hypothesis that targeted activation of ClC-2 Cl\(^{-}\) channels in ischemia-injured intestine would stimulate rapid repair and restoration of mucosal barrier function. Therefore, ischemia-injured porcine intestinal mucosa was mounted on Ussing chambers and indexes of barrier function were assessed in response to treatment with the selective ClC-2 agonist lubiprostone. In line with our hypothesis, mucosal treatment of ischemia-injured porcine ileum and ascending colon with the ClC-2 agonist lubiprostone stimulated rapid recovery of TER and significantly reduced mucosal permeability to the paracellular permeability marker mannitol. During peak recovery of TER in ischemic tissues treated with lubiprostone, occludin was localized exclusively to the TJ, whereas in untreated ischemic tissues occludin staining was diffused. In addition, pretreatment of injured tissues with the ClC-2 blocker ZnCl\(_2\) prevented recovery of TER and occludin restoration. However, it is noteworthy that ZnCl\(_2\) only partially inhibited lubiprostone-stimulated \(I_{sc}\) in ischemic tissues despite recovery of TER to nonischemic control levels. The significance of this effect is unclear, but it may suggest that a portion of the \(I_{sc}\) response evoked by lubiprostone is not required for barrier repair. Previously reported data (37) showed similar findings for PGE\(_2\) and ZnCl\(_2\). However, in the latter study, PGE\(_2\)-evoked \(I_{sc}\) was sensitive to CFTR blockade with DASU-02, whereas in the present study, DASU-02 was without effect on lubiprostone-stimulated \(I_{sc}\), suggesting that CFTR is not a major channel involved in \(I_{sc}\) responses and TER recovery stimulated by lubiprostone. More studies are needed to determine the exact mechanisms by which ClC-2 activation, induced by lubiprostone, stimulates repair of barrier function, especially with regard to the role of epithelial secretion.

The role of ClC-2 in epithelial injury and repair has not been previously investigated. Nonetheless, there is evidence demonstrating ClC-2 activation during cellular stress (1, 20), suggesting that ClC-2 may play an important role in injury and repair processes. In previous studies (37), we demonstrated increased ClC-2 protein expression in ischemia-injured porcine ileal mucosa. In line with our findings, ClC-2 currents in T84 cells and *Xenopus* oocytes were shown to be activated by ATP depletion (20) and actin cytoskeleton disruption (1), respectively, both of which model events during ischemic injury. Recently, heat shock protein 90 has been shown to associate with and enhance ClC-2 activity, which may have important physiological consequences during periods of cellular stress (24).

An interesting characteristic of ClC-2 that may provide insight into the mechanism by which ClC-2 modulates intestinal barrier repair is its localization to the interepithelial TJs (23). CIC-2 localization to this region of the cell may facilitate interactions with TJ proteins and associated regulatory molecules, which, in turn, may regulate the permeability characteristics of the paracellular pathway. In support of this hypothesis, ClC-2 was shown to be critical to the formation of the epithelial barrier in other tissues. For example, the retinal pigment epithelia and seminiferous tubules, both of which require close cell-cell interactions for the establishment of epithelial blood-organ barriers, fail to form properly in ClC-2-knockout mice, resulting in degeneration of the retinal pigment epithelium and testes, respectively (13). We have demonstrated the expression of the TJ occludin in CIC-2 immunoprecipitates in the porcine ileum. In the present study, treatment of injured intestinal tissues resulted in occludin redistribution from the cytoplasm to lateral cellular membrane and TJ. Trafficking and redistribution of TJ proteins from the cytosol to the TJ is a critical component of the resealing process of the TJ and recovery of epithelial resistance (7, 14, 32). The interactions between ClC-2 and TJ proteins during barrier repair require more study.

Activation of ClC-2 with lubiprostone resulted in elevations in \(I_{sc}\) and restoration of pres ischemic TER levels. However, it is not known whether Cl\(^{-}\) transport is critical for repair or whether activation ClC-2 channel alone is required, regardless of whether it transports Cl\(^{-}\). With regard to the former, it is plausible that Cl\(^{-}\) secretion could result in a lumen-directed osmotic gradient serving to pull water from the paracellular space and thus physically collapsing the paracellular space, leading to elevations in TER (11, 30, 33). However, given the marked disruption of the TJ protein architecture that occurs during ischemia in our model, it seems unlikely that an osmotic gradient, resulting from luminal Cl\(^{-}\) accumulation, would form without some degree of TJ integrity. Moreover, inhibition of CFTR-mediated Cl\(^{-}\) currents, which represent the major Cl\(^{-}\) secretory pathway induced by PGE\(_2\) in our model, had no influence on restoration of TER, suggesting that recovery of TER is not a direct result of Cl\(^{-}\) transport. However, it should be noted that the Cl\(^{-}\) uptake inhibitor bumetanide blocked lubiprostone-stimulated \(I_{sc}\) and impaired recovery of TER. This effect may be due to the requirement of intracellular Cl\(^{-}\) for ClC-2 function that has been demonstrated in neuronal and gastrointestinal tissues (15). The latter study showed predominantly basolateral expression of ClC-2 in surface epithelium of the guinea pig colon.

Results from the present experiments demonstrate that targeted activation of CIC-2 Cl\(^{-}\) channels with lubiprostone stimulates repair of intestinal barrier function in the ischemia-injured porcine ileum and colon, associated with alterations in TJ structure. Although prostanoids, particularly PGE\(_2\), are also capable of stimulating a similar reparative function, they appear to be less selective for the reparative process than the prostone lubiprostone. Therefore, from a clinical point of view, lubiprostone may provide a pharmacological method of inducing mucosal repair without undesired side effects such as excessive mucosal secretion and altered motility patterns.
GRANTS

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