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Prostaglandin-mediated closure of paracellular pathway and not restitution is the primary determinant of barrier recovery in acutely injured porcine ileum

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SUMMARY

Small bowel epithelium is at the frontline of intestinal barrier function. Restitution is considered to be the major determinant of epithelial repair as function recovers in parallel with restitution after acute injury. As such, studies of intact mucosa have largely been replaced by migration assays of cultured epithelia. These latter studies fail to account for the simultaneous roles played by villous contraction and paracellular permeability in recovery of barrier function. Non-steroidal anti-inflammatory drugs (NSAID) result in increased intestinal permeability and disease exacerbation in patients with IBD. Thus, we examined the reparative attributes of endogenous prostaglandins (PG) after injury of ileal mucosa by deoxycholate (6 mM) in Ussing chambers. Recovery of transepithelial resistance (TER) from 20–40 $\Omega.cm^2$ was abolished by indomethacin (INDO), whereas restitution of 40–100% of the villous surface was unaffected despite concurrent arrest of villous contraction. In the presence of PG, resident crypt and migrating epithelial cells were tightly apposed. In tissues treated with INDO, crypt epithelial cells had dilated intercellular spaces that were accentuated in the migrating epithelium. TER was fully rescued from the effects of INDO by osmotic-driven collapse of the paracellular space and PG-mediated recovery was significantly impaired by blockade of Cl^- secretion. These studies are the first to clearly distinguish the relative contribution of paracellular resistance versus restitution to acute recovery of epithelial barrier function. Restitution was ineffective in the absence of PG-mediated paracellular space closure. Failure of PG-mediated repair mechanisms may underlie barrier failure resulting from NSAID use in patients with underlying enteropathy.

Keywords

resistance; permeability; tight junction; deoxycholate

INTRODUCTION

The single columnar epithelial lining of the small intestine is the primary barrier restricting transgression by luminal bacteria, endotoxin, bile, digestive enzymes and antigens while also serving as the principal site for selective transport of electrolytes and nutrients. Following

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mucosal injury to the small intestine, acute restoration of barrier function is thought to require the concerted action of three local repair mechanisms. These mechanisms include 1) villous contraction, which reduces the total and denuded surface area for repair (13,26), 2) epithelial cell migration (restitution) to seal exposed basement membrane (1,27), and 3) closure of leaky paracellular spaces and tight junctions between epithelial cells (6,14,21). The relative contribution of each mechanism to early recovery of barrier function is unknown.

Gut barrier failure can be a life-threatening consequence of non-steroidal anti-inflammatory drug (NSAID) use and results in more than 100,000 hospitalizations per year at an estimated cost of $2 billion (17). Substantial information has been gathered concerning the protective attributes of endogenous PG and the role of PG depletion in the genesis of peptic ulcer disease in patients receiving NSAIDs. Considerably less is known about the effects of endogenous PG on barrier function of the small intestine. It appears that simple inhibition of cyclooxygenase is insufficient to cause functional or morphologic injury to small intestinal mucosa. However, in the presence of pre-existing inflammatory bowel disease or luminal aggressive factors such as bile and bacteria, NSAID therapy can be associated with increases in intestinal permeability and disease exacerbation (4,5,12,19,20,33). Accordingly, PG may mediate reparative effects in the small intestine. Nonetheless, little attention has been given to clarifying the local mechanisms of PG action in the small intestine or to determination of whether NSAID result in inadequate repair mechanisms.

We hypothesized that increased synthesis of endogenous PG plays a key role in repair of pre-existing small intestinal injury. Accordingly, we examined the local effects of PG on epithelial repair after deoxycholate-induced injury to porcine ileal mucosa mounted in Ussing chambers. The ileum is a common site for intestinal inflammation, bacterial deconjugation of bile acids, and exposure to high concentrations of enterohepatically recirculated NSAID. After deconjugation, bile acids contribute to injury by triggering increases in tight junction permeability and epithelial cell loss (14). Our *in vitro* model retains the physiologic complexity of interactions between the lamina propria and overlying epithelium while eliminating effects mediated by PG *in vivo* such as leukocyte adhesion, mucosal blood flow, and cell proliferation (16). Consequently, local mechanisms that affect villus contraction, epithelial cell migration and tight junction permeability can be integrated and their contributions to recovery of barrier function quantified.

Results of the present study show that endogenous PG, released upon mucosal injury, mediate local repair of small intestinal epithelium after damage by the deconjugated bile salt, deoxycholate. While ongoing restitution and villous contraction were prominent features of repairing mucosa, acute recovery of barrier function was uniquely dependent on PG-mediated resealing of tight junctions and lateral intercellular space. Failure to repair increases in paracellular pathway permeability may underlie barrier failure resulting from NSAID use in patients with underlying enteropathy.

METHODS

Animals

Six to 8-wk old Yorkshire crossbred pigs were fed a commercial pelleted diet and had free access to water. Pigs were sedated (xylazine 1.5 mg/kg and ketamine 11 mg/kg IM; Fort Dodge, Iowa) and then euthanatized by intravenous pentobarbital injection (Vortech Pharm., Dearborn, MI). Approximately 30 cm of the distal ileum was then removed for *in vitro* studies. All protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Ussing Chamber Studies

Ileal mucosa was separated from the seromuscular layers in an oxygenated (95% O_2 − 5% CO_2) Ringer solution and mounted in 3.14-cm^2 aperture Ussing Chambers. Tissues were bathed on both serosal and mucosal sides by a Ringer solution containing (mmol/L): Na^+, 142; K^+, 5; Ca^{2+}, 1.25; Mg^{2+}, 1.1; Cl^-, 124; HCO_3^-, 25; HPO_4^{2-}, 1.65; and $H_2PO_4^-$, 0.3. The serosal solution contained 10 mM glucose and the mucosal solution contained 10 mM mannitol. Solutions were oxygenated (95% O_2 − 5% CO_2) and circulated in water-jacketed reservoirs maintained at 37°C. Electrical recordings were performed every 15–30-min and included short circuit current (I_{sc}) and spontaneous potential difference (PD). PD was measured using Ringer-agar bridges connected to calomel electrodes. If the PD was between −1.0 and 1.0 mV, tissues were current clamped at ±100 μA for PD measurement. PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrects for fluid resistance (World Precision Instruments; Sarasota, FA). Transepithelial electrical resistance (TER; Ω·cm^2) was calculated from the spontaneous or clamped PD and I_{sc} using Ohms' law.

Isotopic flux studies of epithelial permeability were performed using ^3H-labeled mannitol (2 μCi; 6.6mM; Dupont; Boston, MA) or ^3H-labeled inulin (2 μCi; 0.2mM; Dupont; Boston, MA). Isotope was added to the mucosal reservoir immediately after removal of deoxycholate (time = 30-min). For ^3H-labeled mannitol, three successive 60-min flux periods (from 30 to 210-min) were performed by taking paired samples from the serosal reservoir. For ^3H-labeled inulin, five successive 30-min flux periods (from 60 to 210-min) were similarly performed. Samples were counted for ^3H in a liquid scintillation counter (LKB Wallac; Turku, Finland). Flux of mannitol or inulin from mucosa to serosa was calculated using standard equations.

In Vitro Mucosal Injury

All tissues were allowed to equilibrate in the Ussing chamber for 15-min (time 0–15-min). Deoxycholate (6 mM; Sigma; St. Louis, MO) was then added to the mucosal reservoir for a duration of 15-min (time 15–30-min). Deoxycholate was removed and replaced by Ringer solution and tissue recovery was assessed over a 3-hr period (30–210-min). The 210-min length of study was chosen based on declining viability of uninjured ileum within the Ussing chamber after this time period.

To standardize the severity of injury, tissues having TER <16 or >28 Ω·cm^2 immediately after removal of deoxycholate were discarded from analysis. This injury results in a uniform degree of epithelial damage which is partially repaired during the 3-hr recovery period. Details of our experimental model of acute mucosal injury and repair have been published previously (16). For each pig, uninjured and injured but untreated tissues were included in all experimental designs.

Morphometric Analyses

Tissues were removed from the Ussing chamber after specified time periods. A sample from each Ussing chamber was fixed in formalin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin. Three sections from each tissue were examined without knowledge of prior treatment. Using an ocular micrometer and light microscope, the following measurements were recorded for 5 well-oriented villi and then averaged; 1) villus height measured from the crypt opening to villus tip, 2) crypt depth, 3) villus width at midpoint, 4) linear length of villus perimeter, and 5) linear length of denuded villus. To account for reductions in villus height and surface area that result from stripping and mounting of mucosa (24), measurements were compared to Ussing-chambered, but uninjured tissue, fixed at matching time points.

Villus surface area was calculated using a modified formula for calculating the surface area of a cylinder (3). The formula was modified by subtracting the area of each base of the cylinder,

adding a factor accounting for the hemispherical shape of the apical villus, and multiplying by a factor accounting for the variable position at which each villus is frontally sectioned (22) as follows: villus surface area = (2π • 1/2[(4/π)d] h), where π = 3.14, d = villus diameter (width) at midpoint, and h = villus height.

Determination of the relative contributions of villi and crypts to total luminal surface area was performed as described by Collete *et al* for rat ileal mucosa (10). Briefly, paraffin-embedded tissues were sequentially sectioned in a transverse orientation every 20 μm beginning at the base of the crypts and extending to the villus tips. The crypt surface length was measured in each section from the base of the crypt to its opening at the surface. Surface length of villi were measured from the opening of the crypt to the tip of the villus. Measurements were made using a computer software package (Image-Pro® Plus, Media Cybernetics; Silver Spring, MD). Relative contributions of crypts and villi to total surface area were calculated as follows:

$$\text{Total mucosal area} = \underbrace{\text{crypt surface area}}_{\sum[D \bullet l_c]} + \underbrace{\text{villus surface area}}_{\sum[D \bullet l_v]}$$

D = distance between sections (20μm)

l_c = length of lumen of crypt

l_v = length of periphery of villi

Transmission Electron Microscopy

To examine the ultrastructural effect of endogenous prostaglandins on intercellular space anatomy of deoxycholate-injured porcine ileum, tissues were removed after 210-min in the Ussing chamber and placed in Trump's 4F:1G fixative at 4°C. Samples were processed for transmission electron microscopy using standard techniques (11).

Immunofluorescence Microscopy

Tissues were removed from the Ussing chamber after 210-min, embedded in optimal cutting temperature medium, and frozen-sectioned at 5 μm thickness. Epitope retrieval was performed by boiling the slide-mounted tissue sections in citrate buffer (pH=6) for 10-min. Sections were blocked with 2% BSA prior to incubation with rabbit anti-ZO-1 polyclonal antibody (1:250 in BLOTTO) or isotype control for rabbit primary antibody (Zymed Laboratories; San Francisco, CA) for 2-hrs at room temperature. After rinsing in BLOTTO, sections were incubated with goat anti-rabbit IgG Cy3 conjugate (1:300 in BLOTTO with 2% BSA; Zymed) for 45-minutes in the dark. Sections were rinsed in BLOTTO and PBS, counterstained with DAPI (50 μg/ml; Santa Cruz Biotechnology; Santa Cruz, CA) and coverslipped with aqueous media containing 2.5% DABCO (Sigma; St. Louis, MO). Sections were stored at −20°C. Well-oriented villi were examined using an immunofluorescence microscope.

Eicosanoid Analyses

For eicosanoid analyses, paired samples were taken from the serosal reservoir, gassed with N_2, and frozen in liquid N_2. Samples were stored at −20°C prior to assay. Samples were analyzed for concentration of PGE and 6-keto-PGF1α (the stable metabolite of PGI_2) using commercial ELISA kits according to manufacturer instructions (Biomedical Technologies Inc.; Stoughton, MA).

Assessment of PG Effects on Epithelial Repair

Unless stated otherwise, treatments were added to tissues immediately after deoxycholate removal from the mucosal reservoir (time = 30-min). Treatments included 16,16-dimethyl-PGE_2 (Sigma; St. Louis, MO) and the PGI_2 analogue carbacyclin (Sigma; St. Louis, MO) (each

10^{-6} M serosal), and indomethacin (5×10^{-6} M mucosal and serosal). 16,16-dimethyl-PGE_2 and carbacyclin were examined in combination based on prior studies demonstrating their synergistic action on recovery of barrier function after ischemic injury of porcine ileum (6) and increases in endogenous synthesis of both of these eicosanoids after deoxycholate injury in the present model.

Statistical Analysis

Data are reported as mean ± SE. For all analyses, $P < 0.05$ was considered significant. One-way or repeated-measures ANOVA and a post hoc Tukey's test were used to compare differences between treatment and control tissues. Pearson correlation coefficient was used to examine relationships between TER and morphologic measurements under different treatment conditions. Number of pigs receiving treatment = n.

RESULTS

Local mechanisms of small intestinal repair

Addition of deoxycholate (6 mM for 15-min) to the luminal bathing solution of ileal mucosa mounted in Ussing chambers resulted in an acute decline in transepithelial electrical resistance (TER) and increased ^{3}H-labeled mannitol permeability. After replacement of the deoxycholate with fresh Ringer's solution, there was a gradual and significant recovery of barrier function over a 180-min time interval of repair (Figure 1).

To determine the mechanisms responsible for recovery of barrier function, restitution was quantified by measuring the surface length of denuded and re-epithelialized villus perimeters using an ocular micrometer. Both injured and uninjured tissues were examined after 0, 30, 45, 75, 150 and 210-min in the Ussing chamber (Figure 2A). Immediately after replacement of deoxycholate with fresh Ringer's solution (time = 30-min), there was detachment of enterocytes from the villus tips. Epithelial cell losses continued until time = 75-min. From 75 to 210-min there was ongoing restitution by migrating flattened to cuboidal absorptive cells and a significant decrease in denuded villus surface area (24,951 ± 5,480 μm^2 at 75-min; 10,350 ± 2,702 μm^2 at 210-min; n = 8 each; *$p<0.05$; one-way ANOVA) (Figure 2B). Uninjured tissue remained covered by confluent epithelium for over 210-min if left unperturbed within the Ussing chamber.

Villous contraction was quantified by measurements of villous height taken from injured and uninjured tissues after 0, 30, 45, 75, 150, and 210-min in the Ussing chamber. Deoxycholate injury and repair were associated with a rapid, followed by a gradual phase of villous contraction (Figure 2C). Initial contraction (I; 0–45-min) was acute and inclusive of deoxycholate injury. Subsequent contraction (II; 45–210-min) was ongoing throughout the repair period. Significant crypt contraction also accompanied mucosal repair. Morphometric analysis of injured tissue at 210-min revealed a villous to crypt surface area ratio of 1:1.8 indicating that the epithelialized crypts were responsible for the majority of the total mucosal surface area. Uninjured tissue retained 99.3 ± 13% of initial (post-stripping) villus height, and 101.7±9.5% of initial crypt depth, for over 210-min if left unperturbed within the chamber (n = 4).

Endogenous prostaglandins promote recovery of barrier function

Local synthesis of endogenous prostaglandins (PG) increased significantly after acute deoxycholate injury (PGE: control = 4,410 ± 1,667; injured = 10,218 ± 3,862 pg/ml and PGI_2 [6-keto-PGF1α]: control = 16,769 ± 6,338; injured = 24,714 ± 9,341 pg/ml). Endogenous PG synthesis was inhibited by addition of a non-selective cyclooxygenase blocker immediately

after injury (indomethacin [INDO]; 5×10^{-6}M) (PGE = 496 ± 187; 6-keto-PGF1α = 1,726 ± 653 pg/ml) (n = 7 each at 210-min; ***p<0.001, Student's paired *t* test).

To determine the role of increased PG synthesis in recovery of barrier function, INDO was added to the serosal and mucosal bathing solutions immediately after injury and prior to onset of repair (t = 30-min). Indomethacin abolished recovery of TER (Figure 3A) and increased permeability of the mucosa to ^{3}H-labeled inulin (control = $1.47 \pm 0.13\times10^3$ μmol/cm^2·hr; injured = $3.04 \pm 0.38\times10^3$ μmol/cm^2·hr; injured + INDO = 4.2 ± 0.28 μmol/cm^2·hr at 210-min, n = 7 each; *p<0.05, repeated measures ANOVA). Recognizing the dependence on endogenous PG for recovery of barrier function, we determined whether additional PG could augment repair. Addition of exogenous PG (PGE_2 [10^{-6}M] and carbacyclin [10^{-6}M; an analogue of PGI_2]) to the serosal bathing solution immediately after injury promoted an earlier increase in TER and decreased permeability to ^{3}H-labeled inulin (control = $1.3 \pm 0.11\times10^3$ μmol/cm^2·hr; injured = $3.04 \pm 0.38\times10^3$ μmol/cm^2·hr; PG = 2.5 ± 0.23 μmol/cm^2·hr @ 210-min, n = 9 each; *p<0.05, repeated measures ANOVA) (Figure 3A). Noteably, TER at the end of the 180-min repair period was not different between tissues having endogenous versus exogenous PG exposure. These findings suggested that endogenous PG synthesis was sufficient to mediate the maximal repair response observed at 210-min.

Restitution rate is independent of prostaglandin synthesis—In migration assays of wounded epithelial monolayers, PG appear to either mediate the action of growth factors (36) or trigger the downstream expression of growth factors that promote cell migration (35). To determine if enhanced restitution was responsible for PG-mediated recovery of barrier function in the present model, surface re-epithelialization was quantified in untreated, INDO and PG-treated tissues at 75 and 210-min to examine the basis for differences in TER at these time points (Figure 3B). Neither INDO or PG had any effect on percent re-epithelialization of villi. However, in the presence of endogenous PG, TER appeared to relate positively with measures of epithelial restitution (including denuded villous surface length, calculated denuded villous surface area, and % villous re-epithelialization; p=0.10) while in the absence of PG, no relationship between TER and restitution could be demonstrated (Figure 4). Thus, we could not attribute the effect of PG on TER to an underlying difference in restitution rate.

Endogenous prostaglandins mediate villus and crypt contraction—Prior *in-vivo* studies have demonstrated that PG mediate a modest villous contraction that is insufficient in magnitude to hasten re-epithelialization (13). However, because TER and isotopic permeability *in-vitro* are expressed per unit of serosal surface area (i.e. in reference to the luminal aperture of the Ussing chamber) we sought to examine whether villous contraction was responsible for PG-dependent increases in TER, perhaps by condensing the surface area of the paracellular pathway within the remaining epithelium. Addition of INDO immediately after deoxycholate injury significantly inhibited contraction of both villi and crypts seen during the repair phase. The resulting villi had a greater total surface area involving a proportionate increase in both the denuded and epithelialized surface (Figure 5). Villous contraction was not promoted by the addition of exogenous PG after injury. To determine whether PG additionally mediated the phase of villous contraction seen concurrent with deoxycholate injury, we treated tissues with INDO prior to injury and measured the height of villi immediately after removal of deoxycholate from the Ussing chamber. In the presence of INDO, the initial contractile phase of villi was not inhibited (villous height @ 30-min; injured = 153.3 ± 22.8 μm; n = 8; injured + INDO = 97.4 ± 9.2 μm; n = 7). The difference in TER observed between tissues treated with and without INDO was not related to any measure of mucosal surface amplification (including villus height, villus width, crypt depth, total villous surface length or calculated total villous surface area; p>0.10). Thus, PG-mediated effects on TER could not be attributed to stimulation of villous or crypt contraction.

Contribution of restitution to recovery of epithelial barrier function—We were surprised to observe that in the presence of INDO there was no recovery of TER despite an underlying 73% re-epithelialization of the villi by 210-min. To determine whether a greater degree of epithelialization could promote recovery of TER in the presence of INDO, we stimulated restitution after injury by treating Ussing chambered mucosa with L-arginine (ARG; 5 mM) and fetal bovine serum (FBS; 1%) as previously described (16). In the presence of INDO, ARG+FBS stimulated re-epithelialization of villi from 73 ± 3% to 90 ± 2% despite the greater surface area resulting from concurrent arrest of villous contraction (Figure 6 A,B). Even with restitution over a range of 49–100% of the villus surface, recovery of TER and decreases in ^{3}H-labeled mannitol permeability were not observed in the absence of endogenous PG synthesis (Figure 6 C,D).

Endogenous prostaglandins promote recovery of paracellular permeability— Because PG-dependent recovery of barrier function (210-min; Figure 3A) could not be accredited to effects on restitution rate or villous contraction, we surmised that PG exerted their effects on the paracellular pathway of the epithelium remaining after deoxycholate injury. To examine whether such a mechanism was reparative or could arise simply from effects of PG on normal epithelium, we examined the effect of INDO and exogenous PG on TER and ^{3}H-labeled mannitol permeability of uninjured mucosa mounted in Ussing chambers. Neither INDO or exogenous PG had any effect on TER or permeability when left in contact with uninjured mucosa for over 210-min (control = 57 ± 3.6 $\Omega \cdot cm^2$ and 0.14 ± 0.02 $\mu mol/cm^2 \cdot hr$; PG = 54 ± 4.1$\Omega \cdot cm^2$ and 0.15 ± 0.02 $\mu mol/cm^2 \cdot hr$; INDO = 54 ± 4.3$\Omega \cdot cm^2$ and 0.15 ± 0.01 $\mu mol/cm^2 \cdot hr$ at 210-min, n = 8 each). These results suggest that PG augment recovery of leaky epithelium remaining after acute mucosal injury.

To determine whether a change in paracellular permeability alone was capable of influencing TER by magnitudes similar to that mediated by endogenous PG, we evaluated the effect of osmotic gradient-driven paracellular collapse on recovery of TER after deoxycholate injury. When urea (150 and 300 mOsm) was added to the mucosal reservoir immediately after injury and in the presence of INDO, TER increased to values comparable to that mediated by endogenous PG. Conversely, addition of urea to the serosal reservoir in the presence of PG did not attenuate recovery of TER (Figure 7). The asymmetric responsiveness of TER to osmotic gradients is a reported characteristic of bile salt-induced increases in tight junction permeability and has been attributed to enhanced conductance of the lateral intercellular spaces (14). Because prior studies have shown that PG-mediated tight junction closure is dependent, in part, on Cl^- secretion, we additionally examined the effect of the Cl^- secretion blocker bumetanide (10^{-4} M) on recovery of TER after deoxycholate injury. Bumetanide significantly inhibited recovery of TER in the presence of endogenous PG and without having effects on concurrent restitution or villous contraction (control = 57 ± 3.3; injured = 40 ± 3; injured + BUM = 29 ± 3.8 $\Omega \cdot cm^2$; *$p<0.05$, Student's paired *t* test).

We next sought to examine the effect of PG on the ultrastructural appearance of the tight junction and intercellular space of resident crypt and restituting epithelial cells at peak repair after deoxycholate injury (time = 210-min) (Figure 8). In the presence of PG, the lateral membranes of crypt epithelial cells were tightly apposed. Epithelial cells migrating along the villus remained contiguous, both apically and basally, and were closely adherent to the underlying basement membrane. In tissues treated with INDO however, crypt epithelium showed dilation of the lateral intercellular space below the tight junction. Epithelial cells migrating along the villus maintained only an apical attachment to neighboring cells with expansive separation of the lateral membranes and loss of contact with the basement membrane.

To determine if tight junction organization of the restituting villous epithelium was specifically altered in response to inhibition of PG synthesis, immunofluorescence microscopy for ZO-1

was performed using tissues obtained at peak repair (Figure 9). In uninjured mucosa, ZO-1 was circumferentially localized along the periapical tight junctions. At peak repair, restituting epithelium showed continuous periapical ZO-1 labeling along the sides of the villi that became localized to junctional foci as cells dispersed along the villus tip. In contrast, mucosa treated with INDO revealed ZO-1 labeling with relatively diminished intensity and continuity.

DISCUSSION

Restitution is considered to be a major determinant of early recovery of gastrointestinal barrier function following an acute injury. This conclusion derives from numerous *in vitro* studies demonstrating that barrier function recovers in parallel with morphologic restitution after acute mucosal injury (27,31,34). Consequently, studies of gastrointestinal repair using intact mucosa have largely been replaced by migration assays of wounded epithelial monolayers (8,25,28, 36). Regretably, these latter studies fail to account for the simultaneous roles played by villous contraction and tight junction permeability in recovery of small intestinal barrier function. For the present report we performed a detailed study of the independent and integrated contributions of restitution, villous contraction and paracellular permeability to early recovery of barrier function using an *in vitro* model of acute small intestinal mucosal repair. Results of these studies are the first to demonstrate that restoration of normal paracellular resistance and not restitution or villous contraction was the primary determinant in PG-mediated early recovery of barrier function.

This conclusion is based on the following observations. First, while recovery of TER over time was paralleled by ongoing restitution as seen in prior studies, villous re-epithelialization over a range of 49–100% was incapable of promoting recovery of barrier function in the absence of PG. Second, recovery of TER was not related to a decrease in total mucosal surface area. Third, in the absence of PG, independent manipulation of paracellular permeability with osmotic pressure gradients reproduced the TER measurements obtained in the presence of PG and without concurrent effects on restitution or villous contraction. Finally, paracellular space closure was mediated by an increase in endogenous PG synthesis after injury. Our finding that PG had no effect on restitution has been observed in other acute studies of intact mucosa (2, 7,12). These results contrast those of PG effects on cell migration of wounded epithelial monolayers and may relate to the time required for induction of growth factor synthesis, which exceeds the duration of studies of early mucosal repair (< 4 hrs).

Nevertheless, it is surprising that unimpeded restitution contributed little to early recovery of barrier function or conversely how any recovery of TER was possible in the presence of frank epithelial denudation. Deliberation of these issues necessitates considering the known barrier attributes of simple intestinal epithelia. Transepithelial electrical resistance represents the competence of the epithelium to defend itself against the passive permeation of ions. As such, TER is considered to be the most sensitive measure of epithelial barrier function. There exists two parallel routes by which ions can passively traverse the epithelium. The transcellular pathway entails crossing both the apical and basal plasma membranes of the epithelial cell. The paracellular pathway involves passing through the tight junction and lateral intercellular space (9,23,24,30). Epithelial cell membranes have a high resistance to passive ion flow (1,000 to 10,000 $\Omega.cm^2$) while the overall resistance (TER) of mammalian small intestinal epithelium is only 21 to 100 $\Omega.cm^2$ (30). Accordingly, the major determinant of TER is the integrity of the paracellular pathway which has been calculated to account for 75–94% of the total passive ion flow across small intestinal epithelium (15,29,32). Thus, agents that affect transcellular resistance have little relative effect on TER of these so-called “leaky” epithelia.

An important limitation of TER measurements are their inability to distinguish between changes in tight junction permeability and the presence of epithelial cell loss, both of which

contribute to the overall surface area of low-resistance paracellular pathway. Thus, recovery of TER after acute intestinal injury cannot be attributed to restitution alone without considering the possibility of simultaneous alterations in tight junction permeability; an assessment that requires a detailed dissection of the underlying mechanisms. Further, if early recovery of TER is to be attributed to resealing of tight junctions more so than to restitution, the injury must result in a greater increase in surface area of the tight junctional pathway than the area of denuded epithelium created at the apical villus. The latter is a feasible consideration because 63–73% of the total paracellular conductance of uninjured small intestinal epithelium is via a high linear density of tight junctions residing in the crypts (9,10,24) and following villous contraction, crypt epithelium accounts for the majority of surface area remaining after acute mucosal injury. In order to determine if changes in tight junction permeability could theoretically account for the magnitude of TER observed in our model, we approximated total tight junction surface area using Marcial's measurements of 0.2180 μm paracellular pathway/μm^2 surface area of villus and 0.7680 μm paracellular pathway/μm^2 surface area of crypt (24)(Appendix 1). Given a total denuded and epithelialized villous surface area of 3.5×10^8 μm^2 and 1.2×10^9 μm^2 (i.e. 75% re-epithelialized) and total crypt surface area of 2.75×10^9 μm^2 (64% of the total mucosal surface area) and the theoretical range in width of the tight junction (0.02 – 0.2 μm)(9), yields a range of tight junction surface area from 12 – 58% of the total surface area of low-resistance pathway (tight junction + denuded area). The 2.08-fold difference in total paracellular pathway resulting from changes in tight junction permeability well exceeds the 1.68-fold difference in TER measured in the presence versus absence of endogenous PG synthesis. Accordingly, a PG-induced change in TER due exclusively to closure of the tight junction pathway is a feasible consideration.

Failure to close the intercellular spaces created by migration of epithelium can additionally explain why restitution was incapable of promoting recovery of barrier function in the absence of PG. The intercellular space represents a series component in the pathway of paracellular resistance. Resistance of the intercellular space (R_i) is given by the equation $R_i = \rho L/\omega lp$, where ρ = resistivity of the bulk solution, L = length of the interspace, ω = width of the interspace, and lp = linear amount of paracellular pathway/cm^2 epithelium (9). Under conditions of tight apposition of the lateral membranes (ω = 0.02 μm; (9)) approximations of intercellular space resistance may exceed the specific tight junction resistance depending on the length of the intercellular space. The latter measurement is difficult to accurately estimate due to extensive folding of the lateral membranes lining the intercellular space (Figure 8). However, once the intercellular space exceeds the physiologic range in width of the tight junction (0.02 – 0.2 μm) (9), R_i approaches zero. Thus, a PG-induced closure of intercellular space between resident and in particular, restituting epithelial cells is likely to have also contributed to recovery of TER after deoxycholate injury.

Although we did not conduct a detailed investigation of the mechanism underlying the paracellular effects of PG, prior studies of PG action after ischemic injury of porcine ileum have demonstrated that PGE and PGI_2 act synergistically to promote an osmotic-driven collapse of the tight junction and lateral intercellular space (6). The initiating event appears to involve stimulation of Cl^- secretion that promotes withdrawal of Na^+ and water from the paracellular spaces of the crypt (7). Concurrently, PG inhibit neutral NaCl absorption resulting in decreased paracellular water absorption by the villus. Specific alterations by PG in tight junction structure are suggested by disruption of ZO-1 and occludin localization to the periapical tight junction in ischemia-injured tissues treated with indomethacin (21). Similar mechanisms are likely operative in the present model insofar as synthesis of both PGE and PGI_2 were increased by deoxycholate injury, barrier function could be rescued in the absence of PG synthesis by application of a mucosal osmotic gradient, and PG-mediated recovery was partially inhibited by blockade of Cl^- transport. Because localization of ZO-1 to periapical tight junctions was diminished after treatment of restituting mucosa with indomethacin, it is

likely that a component of the action of PG on paracellular permeability in the present model involves restoration of normal tight junction structure.

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Appendix 1

Calculation of Villus and Crypt Surface Areas

Total villous surface area was calculated using the measured values of **1)** average surface area of each villus, **2)** number of villi per unit serosal surface and **3)** size of the Ussing chamber aperture (314 mm^2). The area of epithelialized and denuded villous surface were derived based on determination that villi were 75% re-epithelialized at 210-min.

Measure @ 210-min	**Deoxycholate injury**
Average villus surface area (VSA) (n = 27)	49863 ± 2334 μm^2
# villi per mm^2 (n = 20)	99 ± 7
Total villous surface area = (VSA)•(# villi per mm^2)•(314mm^2)	1.55×10^9 μm^2
-Epithelialized villous surface area = (0.75)(1.55×10^9 μm^2)	1.2×10^9 μm^2
-Denuded villous surface area = total VSA– epithelialized VSA	3.5×10^8 μm^2

Total crypt surface area was derived after determination that crypts contributed 64% and villi 36% to total mucosal surface area of deoxycholate-injured tissue after 210-min in the Ussing chamber.

$$\underbrace{\text{Total muscosal surface area}}$$

Total crypt surface area = (total VSA/0.36) – total VSA	2.75×10^9 μm^2

Calculation of Surface Area of Tight Junction Pathway

To estimate the surface area of tight junction pathway, we adopted the formula for linear density of tight junction pathway (lp; $\mu m/\mu m^2$) as defined by Claude (9) and measurements taken by Marcial (24) of crypt and villus epithelium from Guinea pig ileum.

Measure @ 210-min	**Epithelialized Villus**	**Crypt**
Density of tight junction pathway (lp; (9))	0.2180 $\mu m/\mu m^2$	0.7680 $\mu m/\mu m^2$
Length of tight junction pathway = lp • (surface area)	2.6×10^8 μm	2.1×10^9 μm
Sum of tight junction length (villus + crypt)	2.4×10^9 μm	
Surface area of tight junction pathway (villus + crypt) = tight junction length • tight junction width (0.02–0.2 μm)	$4.8 \times 10^7 – 4.8 \times 10^8$ μm^2	

Comparison of Contributions of Tight Junction Versus Denuded Surface Area to Total Low-Resistance Paracellular Pathway

Measure at 210-min	**Surface area (%) contribution to total low-resistance paracellular pathway**	
Tight Junction Width (9)	**0.02 μm**	**0.2 μm**
Denuded villous surface area	3.5×10^8 μm^2 (88%)	3.5×10^8 μm^2 (42%)
Tight junction surface area	4.8×10^7 μm^2 (12%)	4.8×10^8 μm^2 (58%)
Total low-resistance paracellular pathway	3.98×10^8 μm^2	8.3×10^8 μm^2
Tight junction-mediated change in surface area of total paracellular pathway	2.08X	

Measure at 210-min	Surface area (%) contribution to total low-resistance paracellular pathway
PG-mediated change in TER	1.68X

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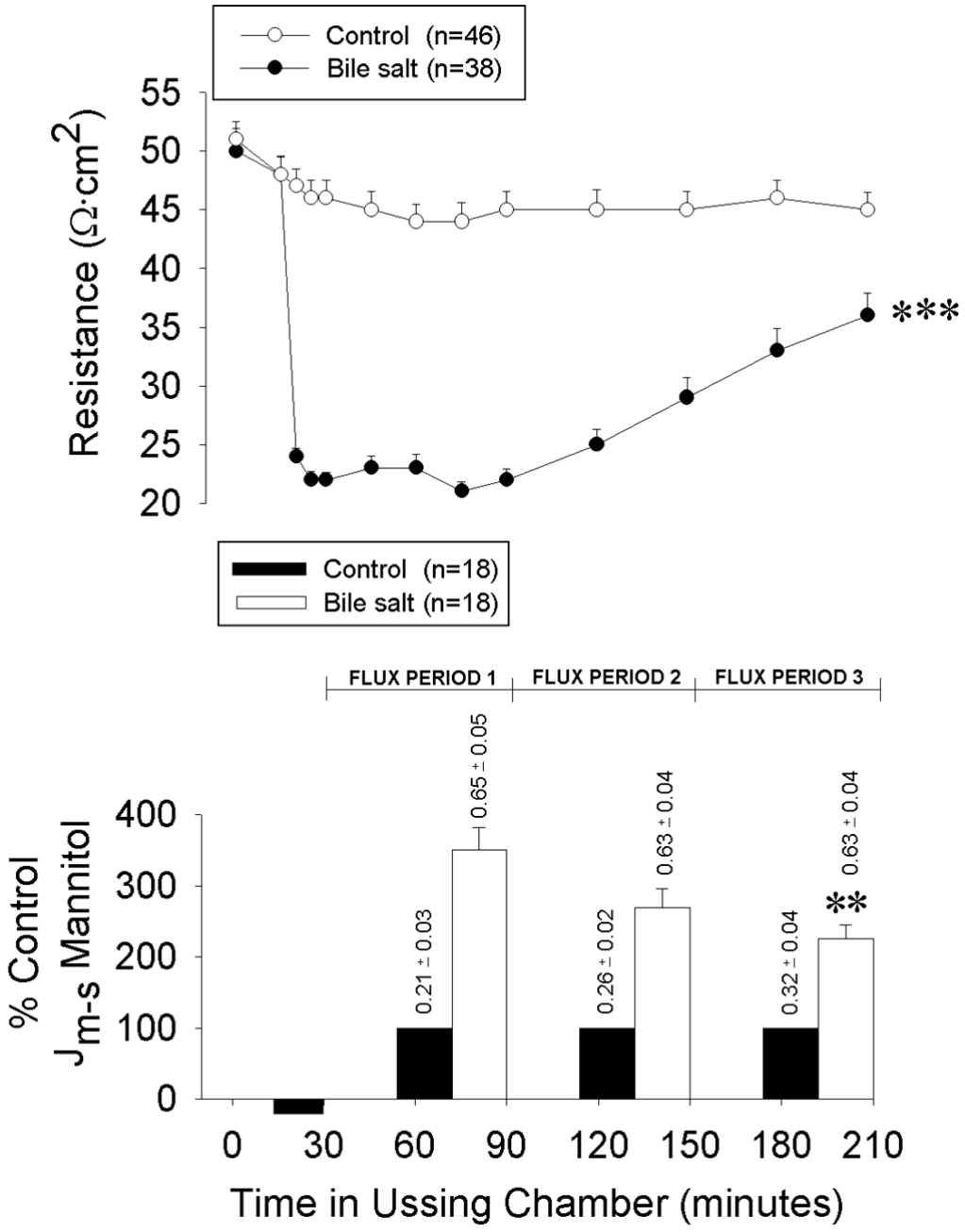

Figure 1.

Transepithelial electrical resistance and mucosal-to-serosal flux ($J_{m\rightarrow s}$) of ^{3}H-labeled mannitol across porcine ileal mucosa mounted in Ussing chambers. Mucosae were acclimated for 15-min followed by a 15-min luminal exposure to 6 mM deoxycholate (black bar). Deoxycholate was then replaced by fresh Ringer's solution (time = 30-min) and recovery of resistance and permeability were recorded over the following 180-min period (***$p<0.001$; two-way repeated measures ANOVA)(** $p<0.01$ compared to injured mucosa at flux period 1 [values above bars indicate raw data]; one-way ANOVA). Values represent mean ± SE; n = number of pigs.

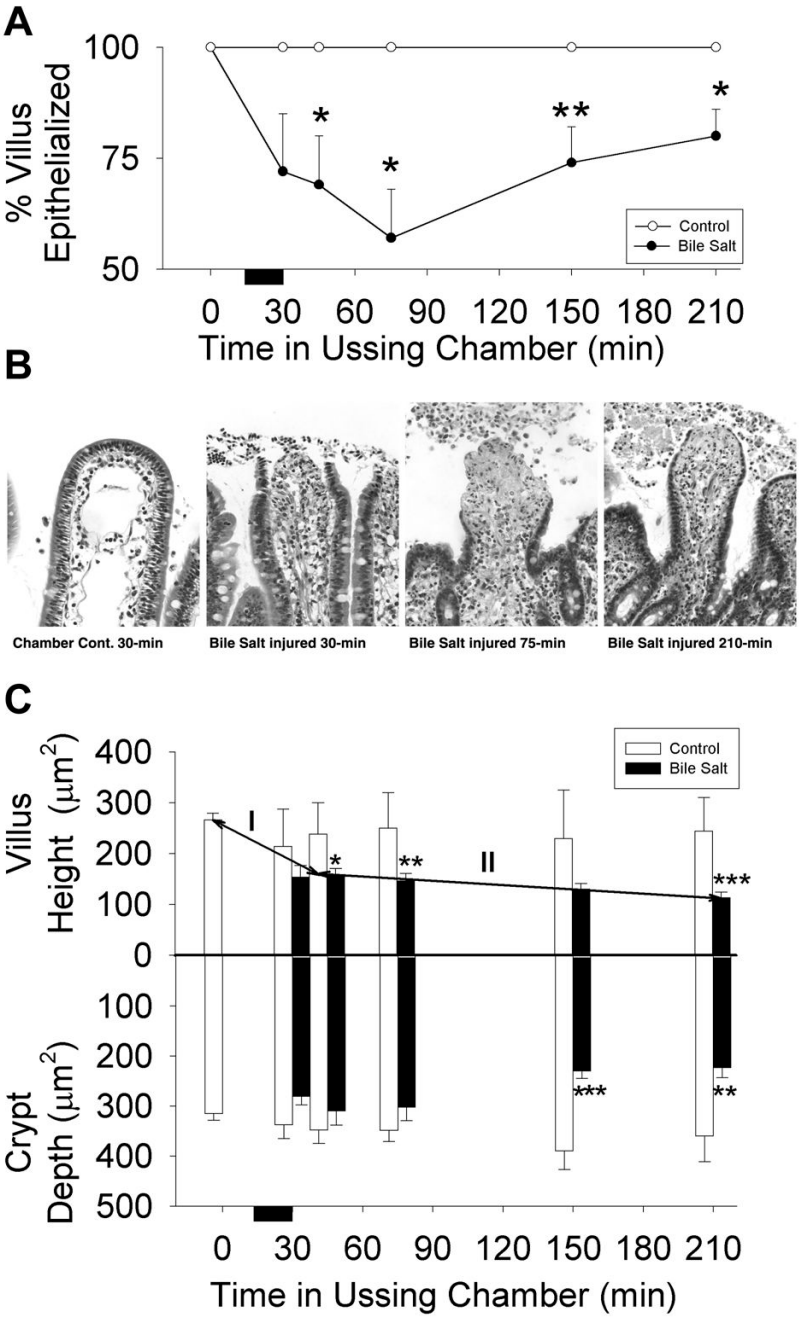

Figure 2.

Restitution and villous contraction of deoxycholate injured porcine ileal mucosa mounted in Ussing chambers. **(A)** Percent villous epithelialization of mucosae removed from the Ussing chamber at specified time periods after deoxycholate injury. Bars represent mean ± SE; n = 4 pigs at 45 and 150-min; n = 8 pigs at 0, 75, and 210-min. (✱p<0.05,✱✱ p<0.01 –vs- time-matched, Ussing-chambered and uninjured control tissue (n = 4 each) (one-way ANOVA). **(B)** Histologic appearance of villi after removal from the Ussing chamber. Commensurate with deoxycholate injury (t = 30-min) there were epithelial losses from the tips of villi. Epithelial losses continued for the first 45-min of recovery culminating in peak injury at time = 75-min. Between 75 and 210-min there was partial restitution of the injured villi by flattened to cuboidal

migrating enterocytes. Chamber-mounted, uninjured control tissue maintained epithelial continuity throughout the study period (chamber control). Magnification, 314X. **(C)** Villus and crypt measurements performed at specified time periods after deoxycholate injury. Bars represent mean ± SE. For injured tissue, n = 4 pigs at 45 and 150-min; n = 8 pigs at 30, 75, and 210-min. ✱ $p<0.05$, ✱✱ $p<0.01$, ✱✱✱ $p<0.001$–vs- time-matched, Ussing-chambered and uninjured control tissue (n = 18 at 0-min and n = 4 at each subsequent time period)(one-way ANOVA). There was a significant decrease in villous height of injured tissue between 45 and 210-min (II), ✱ $p<0.05$ (one-way ANOVA).

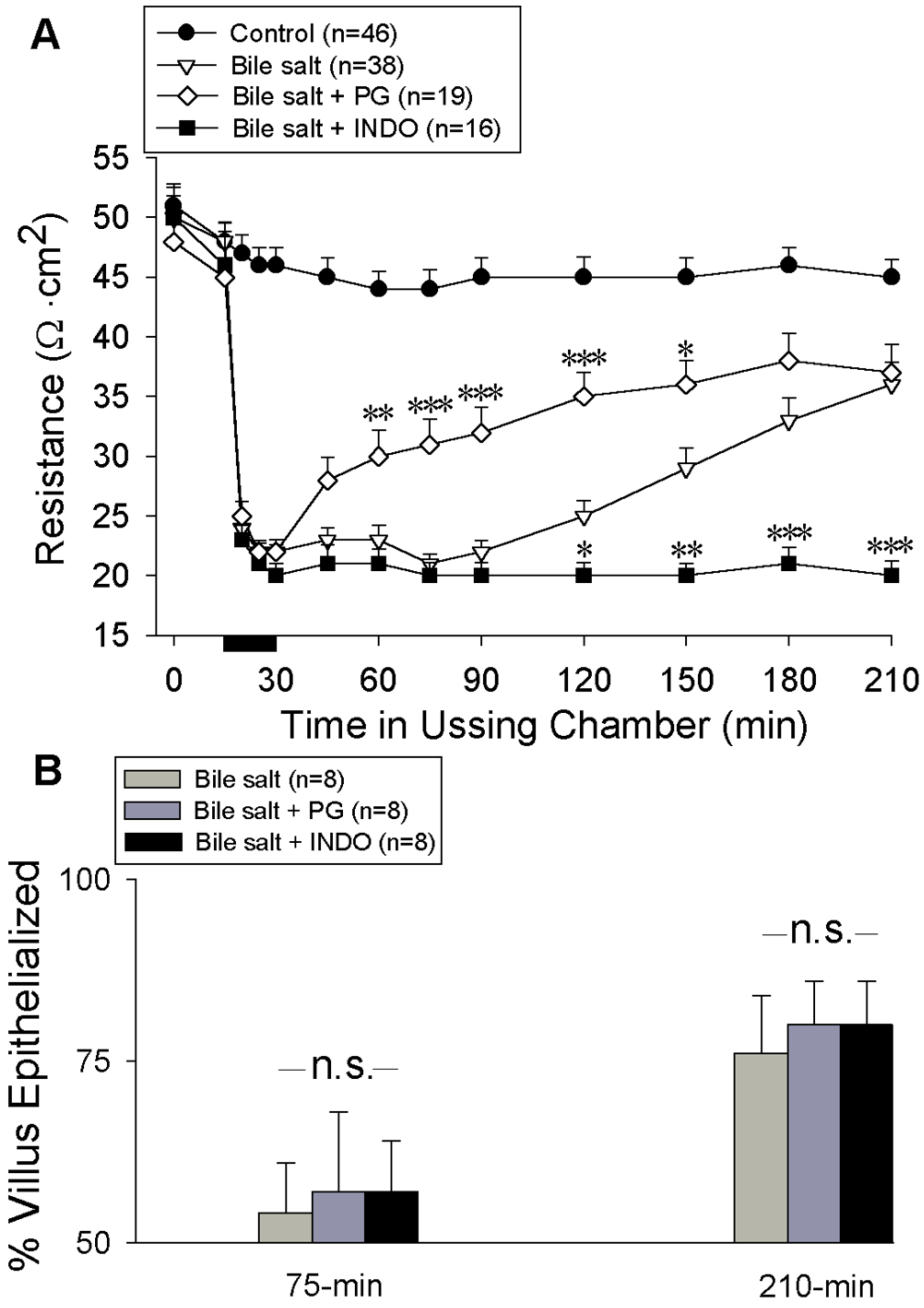

Figure 3.

Recovery of transepithelial electrical resistance **(A)** and restitution **(B)** of Ussing chambered porcine ileal mucosa after deoxycholate injury and treatment with exogenous prostaglandins (PG) or indomethacin (INDO). PGE_2 (10^{-6} M) and prostacyclin (10^{-6} M) or INDO (5×10^{-6}M) were added immediately after injury and prior to onset of repair (time = 30-min). **(A)** INDO abolished recovery of TER while addition of exogenous PG resulted in an early elevation in TER that did not exceed baseline recovery at 210-min (✱ $p<0.05$ ✱✱ $p<0.01$ and ✱✱✱ $p<0.001$ –vs- injured control; one-way ANOVA). **(B)** Percent epithelialization of villi are shown at timepoints corresponding to maximal effects of exogenous PG (75-min) and

INDO (210-min) on recovery of TER. Values represent mean ± SE; n= number of pigs; n.s. = not statistically significant.

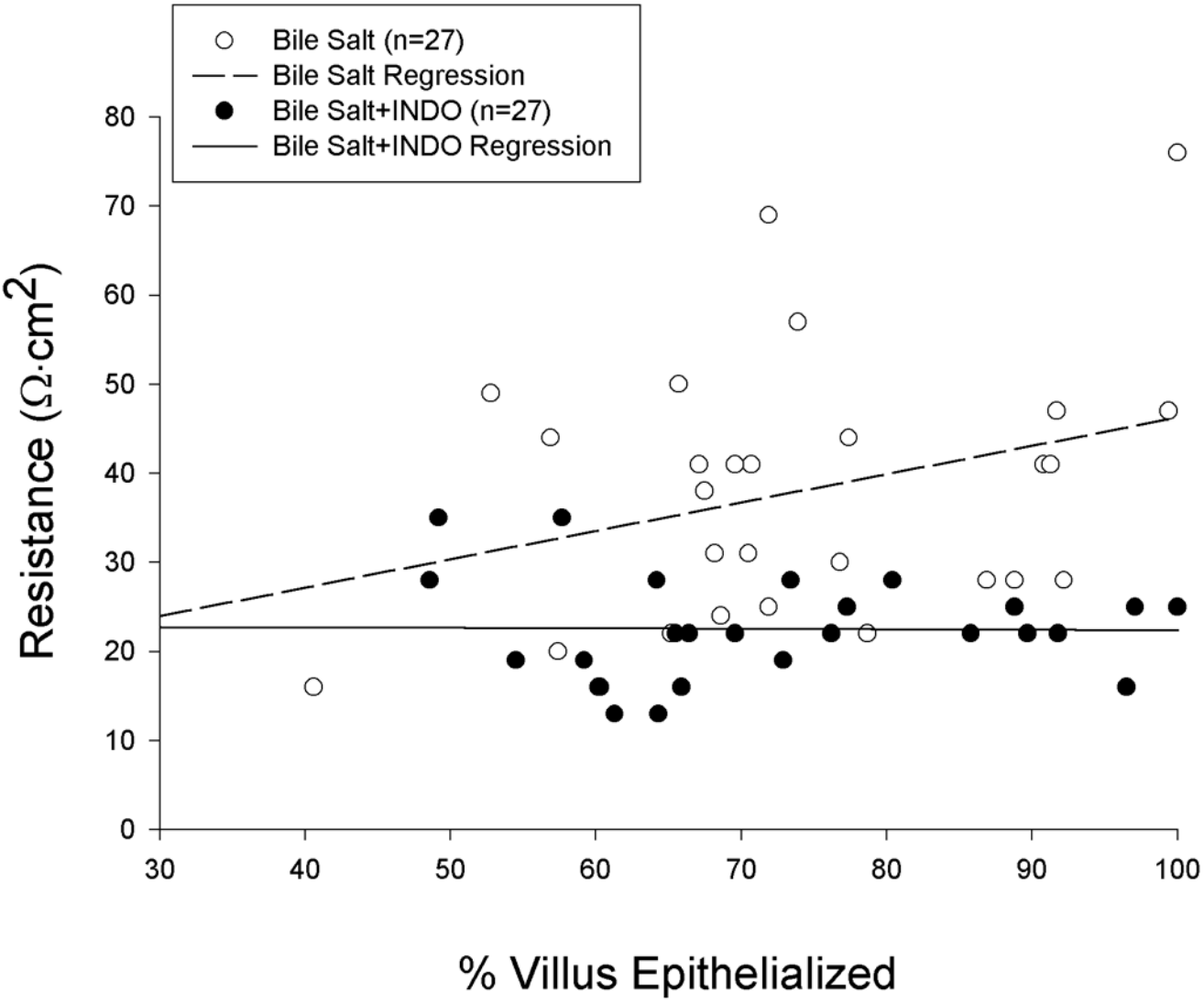

Figure 4.

Transepithelial electrical resistance and degree of restitution attained by porcine ileal mucosa at peak repair (time = 210-min) after injury by deoxycholate in Ussing chambers. Although restitution was equivalent in the presence or absence of INDO (5×10^{-6}M), there was no correlation between TER and restitution among mucosae treated with INDO ($p>0.10$; Pearson correlation coefficient).

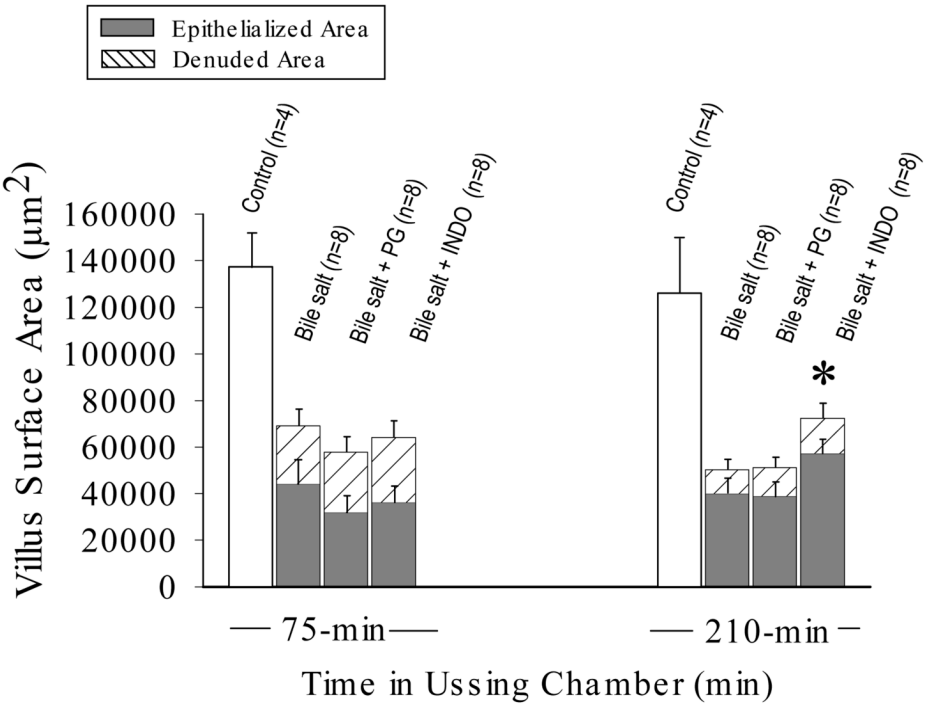

Figure 5.

Relative contributions of epithelialized and denuded villus to total villous surface area of deoxycholate-injured mucosae at peak injury (time = 75-min) and maximal repair (time = 210-min). Indomethacin (INDO; 5×10^{-6}M) arrested villous contraction during repair, resulting in a significant increase in total villous surface area compared to injured control or prostaglandin-treated (PG) tissue at 210-min (* $p<0.05$; one-way ANOVA). Restitution (% villous epithelialization) was unaffected by either treatment. Values represent mean ± SE; n = number of pigs.

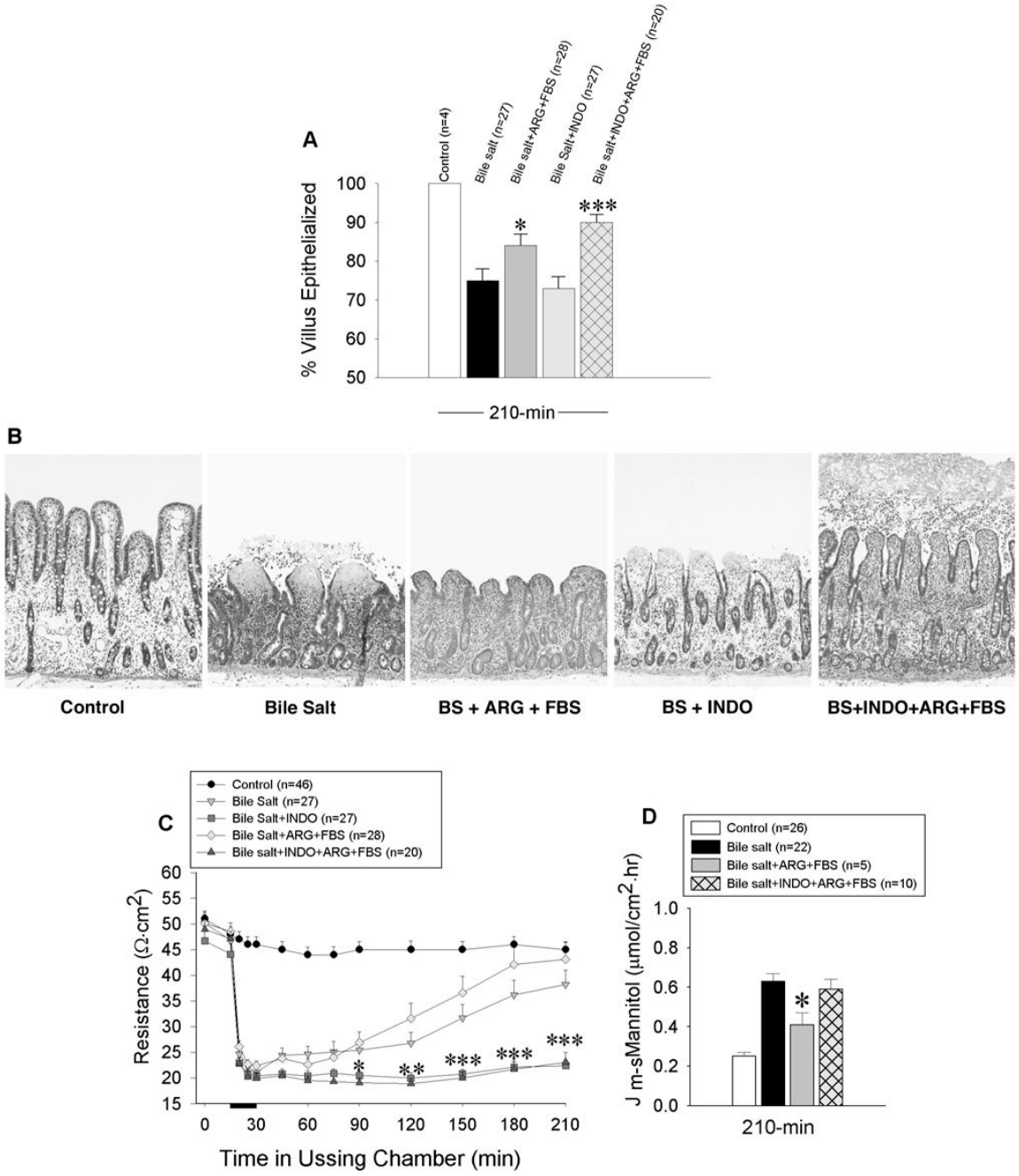

Figure 6.

Effects of enhanced restitution on recovery of barrier function after deoxycholate injury to porcine ileal mucosa mounted in Ussing chambers. Restitution was stimulated by addition of L-arginine (ARG; 5 mM) and fetal bovine serum (FBS; 1%) to the serosal and mucosal reservoir immediately after injury and prior to onset of repair. Results are shown for mucosae at peak repair (**A, B**). Indomethacin (INDO; 5×10^{-6}M) did not attenuate baseline or stimulated restitution despite arrest of villus contraction during recovery. Magnification, 250X. Barrier function, as determined by measures of transepithelial electrical resistance (TER)(**C**) and mucosal to serosal flux of ^{3}H-mannitol ($J_{m\rightarrow s}$) (**D**), was abolished in the absence of endogenous

prostaglandin (PG) synthesis, despite nearly complete restitution of villi. (✱p<0.05, ✱✱p<0.01, ✱✱✱p<0.001; one-way ANOVA). Values represent mean ± SE; n = number of pigs. Bar = bile salt exposure.

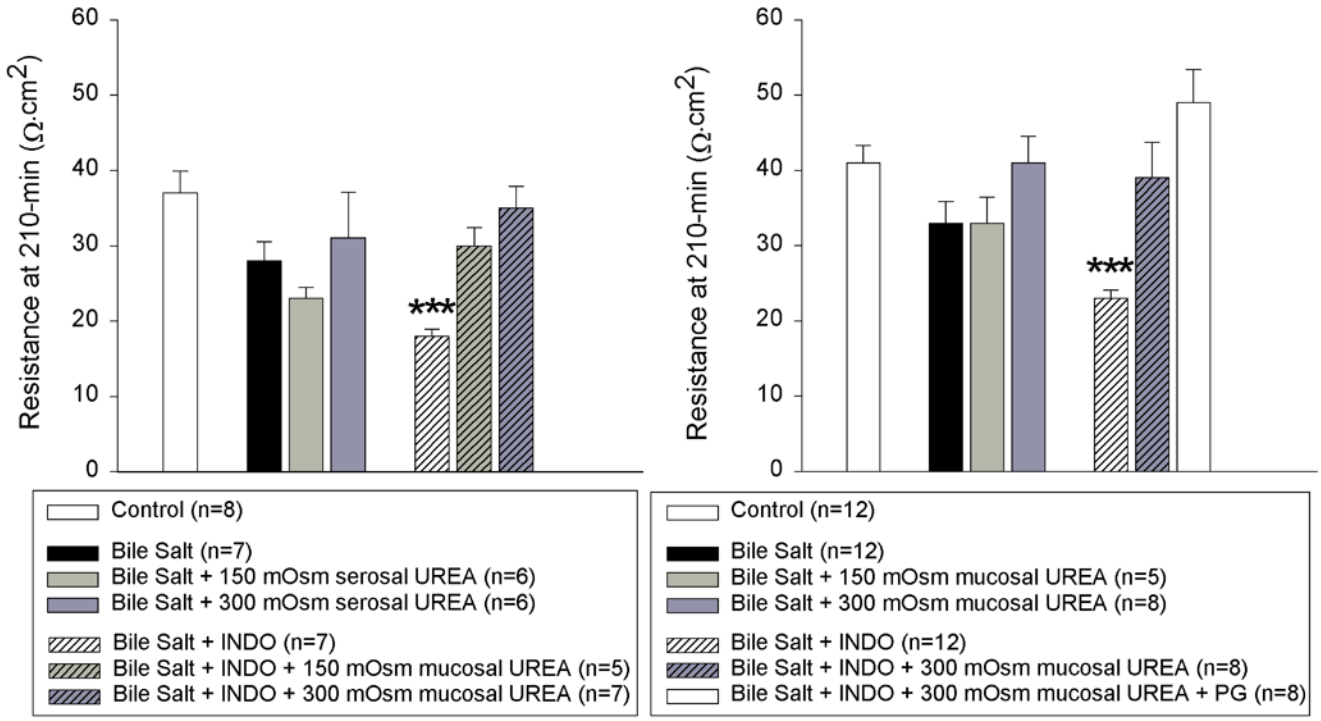

Figure 7.

Transepithelial electrical resistance (TER) of porcine ileal mucosa at peak repair (time = 210-min) after injury by deoxycholate in Ussing chambers. A mucosal to serosal osmotic gradient of urea did not attenuate PG-mediated recovery of TER. Conversely, a serosal to mucosal osmotic gradient of urea could reproduce the effect of endogenous PG on recovery of TER after deoxycholate injury. Values represent mean ± SE; n = number of pigs. (***p<0.001; one-way ANOVA).

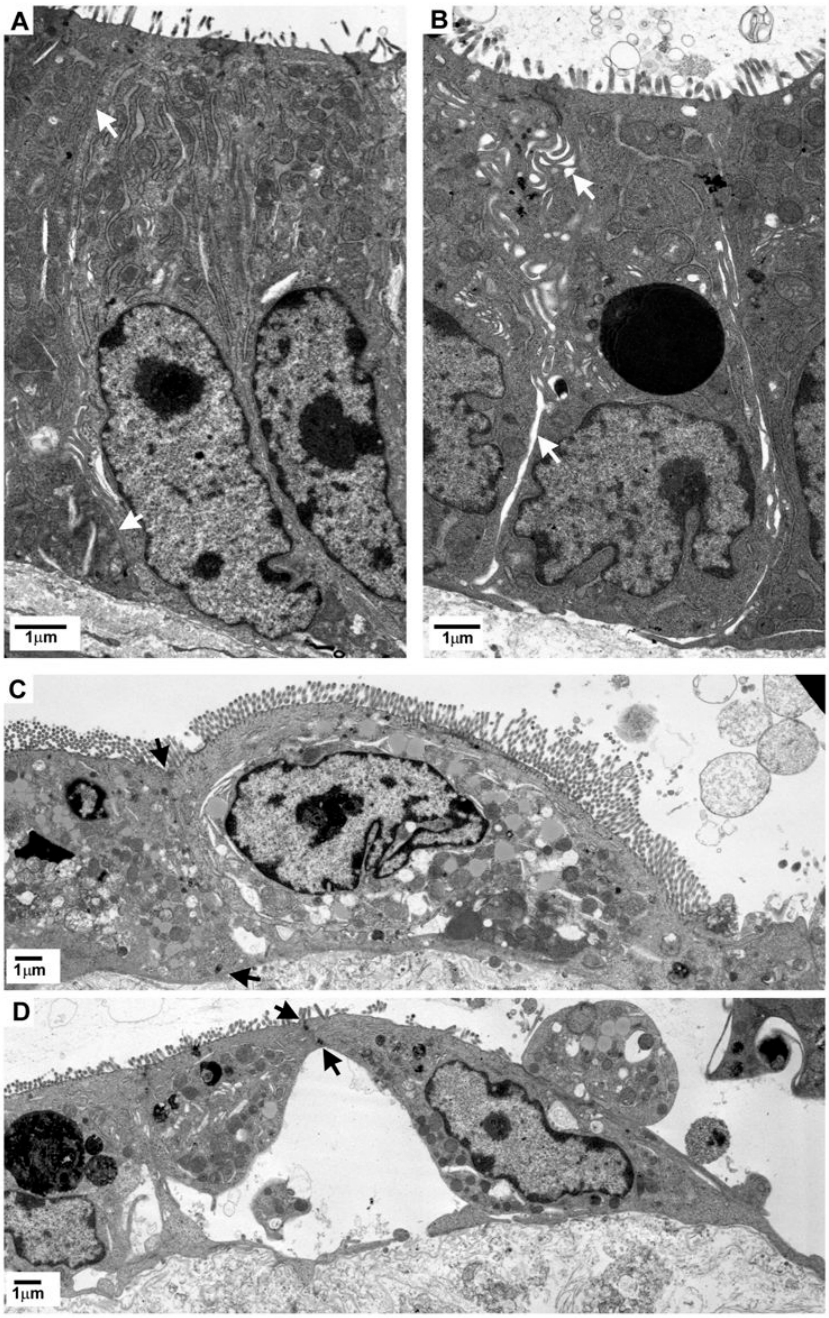

Figure 8.

Transmission electron micrographs of crypt (**A&B**) and migrating villous enterocytes (**C&D**) from porcine ileal mucosa at peak repair after deoxycholate injury (time = 210-min). Mucosae shown in **B & D** were treated with indomethacin (INDO; 5×10^{-6}M) immediately after injury and prior to onset of repair. White arrows indicate intercellular space. Black arrows indicate sites of lateral membrane attachment between adjacent, migrating enterocytes.

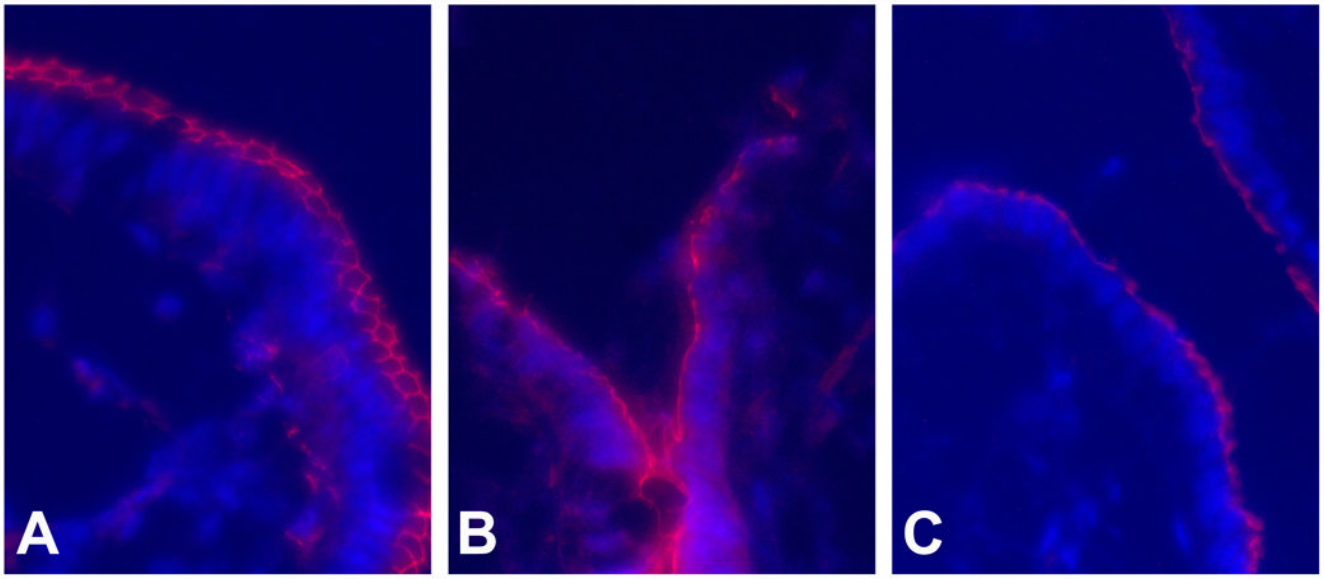

Figure 9.

Immunofluorescence localization of the junctional complex protein ZO-1 in porcine ileal mucosa. (**A**) Uninjured villous mucosa. (**B**) Villous mucosa at peak repair after deoxycholate injury (time = 210-min). (**C**) Villous mucosa treated with indomethacin (INDO; 5×10^{-6}M) immediately after deoxycholate injury and examined at peak repair (time = 210-min). Magnification, 314X.