Gastro protective properties of the novel prostone SPI-8811 against acid-injured porcine mucosa

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Abstract

AIM: To evaluate the protective properties of novel prostone CIC-2 agonist SPI-8811 in porcine model of gastric acid injury.

METHODS: Porcine gastric mucosa was mounted in Ussing chambers and injured by bathing mucosal tissues in an HCl Ringer's solution (pH = 1.5) with or without SPI-8811 (1 μmol/L), cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor (inhibitor 172, 10 μmol/L, apical) and CIC-2 inhibitor ZnCl2, 300 μmol/L, apical), on the apical surface of tissues. Transepithelial resistance and mucosal-to-serosal 3H-mannitol fluxes were measured over a 90-min period. Tissues were analyzed by morph metric techniques, Immunofluorescence and by western blots.

RESULTS: Compared with control tissues, acid exposure decreased transepithelial electrical resistance (TER) and increased 3H-mannitol flux. Pretreatment of gastric mucosa with SPI-8811 was protective against acid-induced decreases in TER (TER, 50 Ω·cm² vs 100 Ω·cm²) and abolished increases in flux (3H-mannitol flux, 0.10 μmol/L·cm² vs 0.04 μmol/L·cm²). Evidence of histological damage in the presence of acid was markedly attenuated by SPI-8811. Immunofluorescence and western analysis for occludin revealed enhanced localization to the region of the tight junction (TJ) after treatment with SPI-8811. Pretreatment with the CIC-2 inhibitor ZnCl2, but not the selective CFTR inhibitor 172, attenuated SPI-8811-mediated mucosal protection, suggesting a role for CIC-2. Prostone may serve both protective and reparative roles in injured tissues.

CONCLUSION: CIC-2 agonist SPI-8811 stimulated enhancement of mucosal barrier function by protecting TJ protein occludin in porcine gastric mucosa and thus protected the gastric acid injury in porcine stomach.

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Key words: Stomach; Mucosal permeability; CIC-2 chloride channel; Tight junction

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INTRODUCTION

It is becoming increasingly evident that many patients suffer from gastric ulcers, particularly in groups of pa-
patients such as those in intensive care facilities\(^{[1-4]}\). This suggests that medications that provide gastro protection have the potential to reduce morbidity associated with gastric ulceration. For decades, agents that suppress acid secretion\(^{[5-7]}\) have been widely used for treatment of gastric ulcers\(^{[8-10]}\). Gastric ulcer disease and repair is complex, involving inflammation, cell proliferation, formation of granulation tissue, and angiogenesis\(^{[11,12]}\). However, gastro protection has also been studied in depth. For example, studies with rebamipide or misoprostol, geranylgeranyl hydrochloride (HSP70) have revealed that these compounds have gastroprotective properties as well enhancing ulcer healing\(^{[13,14]}\). Nonetheless, our understanding of the mechanisms of gastro protection is incomplete and there is the prospect of novel pharmacological agents, aside from antacids, proton pump inhibitors, and prostanoid activators or analogs, which might protect the stomach\(^{[15,16]}\).

The gastric barrier is composed of a single layer of columnar epithelium, mucus, bicarbonate layer, and intraepithelial tight junctions (TJs) residing at the apical-most region of the paracellular space. The gastric barrier serves as the first line of defense against a hostile luminal environment. The mucus bicarbonate barrier is the only pre epithelial barrier between lumen and the epithelium\(^{[17,18]}\). When it is overwhelmed or breaks down in disease, the next series of protective mechanisms come into play, including intracellular neutralization of acid, rapid epithelial repair, and maintenance and distribution of mucosal blood flow. There are two components of this innate mucosal defense: mechanisms that reduce the ability of pathogens and their toxins to invade the mucosa, and the mechanisms that ensure rapid repair of defects in the epithelial monolayer\(^{[19,20]}\). The next line of mucosal defense is formed by a continuous layer of surface epithelial cells which secrete mucus and bicarbonate and generate prostaglandins, heat shock proteins, refoi factor peptides, and cathelicidins. Because of the presence of phospholipids on their surfaces, these cells are hydrophobic, repelling acid- and water-soluble damaging agents. Interconnected by TJs, the surface epithelial cells form a “barrier” preventing back diffusion of acid and pepsin. Once the epithelial barrier is disrupted, epithelial repair mechanisms must rapidly re-form a continuous epithelial monolayer in order to prevent entry of protons and bacteria\(^{[21,22,23]}\). The remarkable phenomenon of epithelial restitution during which epithelium re-seals mucosal defects in the presence of acidic environment has been a subject of several studies.

Acid injury is an important component of gastric ulcer disease, despite more recent findings on the role of Helicobacter pylori which also contributes to this trouble-some problem. Aside from injuring epithelium directly, acid causes disruption of interepithelial TJ protein complexes thereby increasing epithelial permeability\(^{[24,25,26]}\). Models assessing the mechanisms of intestinal injury have demonstrated that the critical event defining disruption of barrier function is the loss of TJ architecture and redistribution of TJ proteins such as occludin from the apical region of the interepithelial space to the cytosol\(^{[27,28,29]}\). In our previous work, we have demonstrated a critical role for Cl secretion in restoration of intestinal barrier function in ischemic-injured porcine ileum\(^{[21]}\). This appears to be attributable to activation of the Cl channel ClC-2, which is localized to the TJ\(^{[22]}\). More specifically, prior studies have shown that the nonselective secretory agonist prostaglandin E2 triggered rapid recovery of transepithelial electrical resistance (TER) and reduced mucosal-to-serosal fluxes of \(^1\)H-mannitol in ischemic-injured intestinal mucus\(^{[20,22-24]}\). Previous studies have shown that activation of ClC-2 by the prostone Lubiprostone\(^{[22,25]}\) enhanced recovery of barrier function in ischemic-injured porcine intestine. Cobiprostone (SPI-8811) is an investigational ClC-2 agonist prostone compound under development by Sucampo Pharmaceuticals Inc. (Bethesda, MD). Prostanes are derived from fatty acids that are formed naturally within tissues. The present study was performed to evaluate the ability of the novel ClC-2 agonist SPI-8811 to provide gastro protection in acid-injured porcine gastric mucosa.

**MATERIALS AND METHODS**

**Chemicals**

ZnCl\(_2\), Bumetanide, \(^1\)H-mannitol, and cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor 172 were purchased from Sigma-Aldrich (St. Louis, MO). SPI-8811 was provided by Sucampo Pharmaceuticals Inc., Bethesda, MD.

**Experimental animals**

All studies were approved by the North Carolina State University Institutional Animal Care and Use committee. Yorkshire crossbred pigs of either sex approximately 10-15 kg body weight were housed individually and maintained on a commercial pelleted feed. Pigs were held off feed, but had free access to water, for 12 h before each experiment. Anaesthesia was induced with xylazine (1.5 mg/kg, im) and ketamine (11 mg/kg, im), after which they were euthanized with pentobarbital (20 mg/kg, iv). The entire stomach was clamped proximally and distally with Doyen intestinal forceps.

**Ussing chamber studies**

After harvesting the entire stomach, it was sharply incised at the lesser curvature and washed in porcine Ringer’s (mmol/L: 154 Na\(^+\), 6.3 K\(^+\), 137 Cl\(^-\), 0.3 H\(_2\)PO\(_4\), 1.2 Ca\(^2+\), 0.7 Mg\(^2+\), 24 HCO\(_3\), pH 7.4) and maintained in oxygenated (95% O\(_2\)/5% CO\(_2\)) Ringer’s solution. The fundus portion was isolated for Ussing chamber studies. After stripping the mucosa from the seromuscular layer by using blunt scissors, mucosa was mounted in 3.14 cm\(^2\) aperture Ussing chambers. Gastric mucosa from individual pigs was mounted on multiple Ussing chambers and subjected to acid injury and select treatments. Tissues were initially bathed in 10 mL porcine Ringer’s on both mucosal and serosal sides. The serosal bathing solution contained 10 mmol/L glucose to maintain tissue viability,
and this was osmotically balanced on the mucosal side with 10 mmol/L mannitol. Indomethacin (5 μmol/L) was added on the serosal and mucosal sides of gastric tissues to prevent prostaglandin production. Bathing solutions were oxygenated and maintained at 37 °C by water jacketed reservoirs. The spontaneous potential difference (PD) was measured via Ringer’s-agar bridges connected to calomel electrodes, and the PD was short-circuited via Ag-AgCl electrodes using voltage clamps that corrected for fluid resistance to measure short circuit current (Isc). Transepithelial resistance (Ω·cm²) was calculated from the spontaneous PD and Isc. If the spontaneous PD was between -1.0 mV 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 90 min experimental period.

**Experimental treatments**

Once tissues were mounted on Ussing chambers, treatments aimed at inhibiting CIC-2 (ZnCl₂, 300 μmol/L, apical), CFTR (inhibitor 172, 10 μmol/L, apical), or Na⁺-K⁺-2Cl⁻ cotransporter (NKCC1) ( bumetanide, 100 μmol/L, basolateral) were added. Alternatively, SPI-8811 was administered to the apical surface of tissues (1 μmol/L) to enhance epithelial Cl⁻ secretion via CIC-2. Following a 30 min equilibration period, HCl (1 mol/L in Ringer’s) was added to the mucosal surface of the tissue to induce acid injury. The pH was monitored with a pH meter (Hanna Instruments Inc., Ann Arbor, MI, United States) and maintained the pH at 1.5 by adding 1 mol/L HCl as needed during the experiment.

**Mucosal-to-serosal fluxes of ³²H-mannitol**

To assess mucosal permeability, 0.2 Ci/mL ³²H-mannitol was placed on the mucosal side of tissues after experimental treatments. After a 15 min equilibration period, standards were taken from the mucosal side of each chamber and a 30 min flux period was established by taking 0.5 mL samples from the serosal compartments. The presence of ³²H was established by measuring emission in a liquid scintillation counter (Rack Beta, Perkin Elmer Life and Analytical Sciences, Boston, MA, United States). Unidirectional mucosal-to-serosal ³²H-mannitol fluxes were calculated using a previously established spreadsheet[7].

**Histological analyses**

Tissue samples were collected at 0, 30 and 90 min during the experimental period and fixed in 10% formalin for histological evaluation. Paraffin embedded samples were sectioned (5 μm) and stained with hematoxylin and eosin and periodic acid-schiff’s stain (PAS). For each tissue, mucosal epithelial lining and gastric pits (crypts) were identified to assess the damage caused by acid (pH 1.5).

**Immunofluorescence labeling of occludin**

For this procedure, tissues were embedded in optimal cutting temperature medium, frozen, and sectioned at 5 μm. Tissue sections were blocked with 2% Bovine serum albumin followed by incubation with rabbit anti-occludin polyclonal antibody (1:150, Zymed, San Francisco, CA, United States) overnight at 4 °C. Sections were washed with PBS and incubated for 45 min with FITC-conjugated anti-rabbit secondary antibody. Sections were mounted in fluorescent mounting medium, and well-orientated gastric pits were examined with a photomicroscope linked to a digital camera.

**Gel electrophoresis and Western blotting**

Following Ussing chamber experiments, gastric mucosal samples were snap frozen and stored at -70 °C prior to performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Tissue aliquots were thawed at 4 °C and added to chilled lysis buffer, including protease inhibitors (0.5 mmol/L Pefabloc, 0.1 mmol/L 4-nitrophenyl phosphate, 0.04 mmol/L glycercophosphate, 0.1 mmol/L NaVO₄, 40 μg/mL bestatin, 2 μg/mL aprotonin, 0.54 μg/mL eupeptic, and 0.7 μg/mL pepstatin A) (Sigma-Aldrich Inc., St. Louis, MO, United States) 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 (BCA Protein Assay Kit, Pierce, Rockford, IL, United States). Tissue extracts (amounts equalized by protein concentration) were mixed with an equal volume of 2 × SDS-PAGE sample buffers and boiled for 4 min. Lysates were loaded on an SDS polyacrylamide gradient gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a polyvinylidene fluoride membrane (Immobilon®, Millipore, Billerica, MA, United States) by using an electro blotting minitransfer apparatus. Membranes were blocked at room temperature for 2 h in Tris-buffered saline plus 0.05% tris-buffered saline tween-20 and 5% dry powdered milk, and then incubated overnight in primary antibody at 4 °C in rabbit anti-rat ClC-2 (Alph Diagnostics, San Antonio, TX, United States), rabbit anti-CFTR (Santa Cruz Biotech Inc., CA, United States) or rabbit anti-occludin (Zymed Laboratories Inc., San Francisco, CA, United States). After multiple washings in TBST, membranes were incubated with horseradish peroxidase conjugated secondary antibody, and developed for visualization of protein with luminol enhancer solution (Pierce, Rockford, IL, United States).

For preparation of detergent soluble and detergent insoluble fractions of gastric mucosa, the tissue samples were extracted in lysis buffer (20 mmol/L Tris, 5 mmol/L MgCl₂, 0.3 mmol/L EGTA, 210 μg/mL sodium fluoride, 18.5 μg/mL sodium orthovanadate, 30 mmol/L sodium pyrophosphate, and complete mini Protease inhibitor cocktail tablet (Thermo Fisher Scientific, Rockford, IL, United States). Following brief centrifugation to remove debris, Triton X-100 soluble and insoluble fractions were collected by incubation and centrifugation (50 000 g for 30 min at 4 °C) with lysis buffer containing 0.5% Triton X-100 and 0.5% SDS, respectively. The samples were processed through an SDS sample preparation kit (Ther-
Acid injury model

To establish a gastric acid injury model, porcine gastric mucosa was mounted on Ussing chambers. After an equilibration period of 30 min the mucosal side was subjected to normal Ringer’s (NR) solutions (pH 7.4) or HCl Ringer’s (pH 1.5) over a 90 min period. Extensive epithelial sloughing and erosion extending into the glandular region of the gastric mucosa and sub-epithelium was noted. Tissues pretreated with SPI-8811 had far lesser evidence of mucosal injury in response to acid, although the epithelium appeared to have flattened to maintain the barrier unimpaired. Alternatively, when the gastric mucosa was exposed to acid Ringer’s (pH 1.5) for 90 min. Mucosa subjected to acid injury had significantly lower TER values than compared to the gastric mucosa bathed in NR, indicating electrophysiological evidence of disruption of epithelial barrier function. Alternatively, pretreatment of the apical side of gastric mucosa with the ClC-2 agonist SPI-8811 in the presence or absence of apical SPI-8811. In agreement with TER responses, acid-injured tissues exhibited increases in permeability to H-mannitol as compared to control uninjured tissue. Pretreatment of the apical surface of mucosal tissues SPI-8811 ablated changes in permeability noted in untreated acid-injured tissues (Figure 1B).

Histological and Immunofluorescence microscopic findings in acid-injured gastric mucosa

Gastric mucosa from Ussing chambers after 90 min in NR revealed intact gastric pits with intact epithelial lining. Alternatively, when the gastric mucosa was exposed to acid (pH 1.5) for 90 min. Extensive epithelial sloughing and erosion extending into the glandular region of the gastric mucosa and sub-epithelium was noted. Tissues pretreated with SPI-8811 had far lesser evidence of mucosal injury in response to acid, although the epithelium appeared to have flattened to maintain the barrier and the gastric pits appeared dilated (Figure 2A).

Using PAS staining for mucus we observed that the loss of mucus by acid injury was seen restored by pretreatment of CIC-2 agonist (Figure 2B). In further experiments, we performed Immunofluorescence of occludin to assess the gastric mucosal TJs. As compared to apical immunolocalization of occludin in the control uninjured gastric mucosal lining epithelium, acid-injured tissues revealed loss of occludin immunofluorescence (Figure 2C). In contrast, gastric mucosa pretreated with SPI-8811 and occludin immunofluorescence (Figure 2C).

Figure 1  Barrier function of acid injured porcine gastric mucosa to pre-treatment of SPI-8811 (1 μmol/L). A: Porcine gastric mucosa mounted on Ussing chambers challenged with mucosal acid (HCl, pH 1.5) over a 90 min period to acid blocked reductions in TER (TER, 50 μΩ cm² vs 100 μΩ cm²). Values represent means ± SE, n = 6. As an alternative assessment of gastric mucosal barrier function, mucosal-to-serosal fluxes of H-mannitol were examined. In agreement with TER responses, H-mannitol flux was significantly elevated by treatment with acid (H-mannitol flux, 0.02 μmol/L·cm⁻² vs 0.10 μmol/L·cm⁻²), and this response was inhibited by SPI-8811. (H-mannitol flux, 0.10 μmol/L·cm⁻² vs 0.04 μmol/L·cm⁻²). Values represent mean ± SE, n = 6. Values represent mean ± SE, n = 6. Values represent mean ± SE, n = 6.

Effects of the ClC-2 agonist SPI-8811 on TER and mucosal-to-serosal H-mannitol fluxes in acid-injured porcine gastric mucosa

As previously described, porcine gastric mucosa was mounted on Ussing chambers and the mucosal surface was exposed to acid Ringer’s (pH 1.5) over a 90 min period. Extensive epithelial sloughing and erosion extending into the glandular region of the gastric mucosa and sub-epithelium was noted. Tissues pretreated with SPI-8811 had far lesser evidence of mucosal injury in response to acid, although the epithelium appeared to have flattened to maintain the barrier unimpaired. Alternatively, when the gastric mucosa was exposed to acid Ringer’s (pH 1.5) for 90 min. Mucosa subjected to acid injury had significantly lower TER values than compared to the gastric mucosa bathed in NR, indicating electrophysiological evidence of disruption of epithelial barrier function. Alternatively, pretreatment of the apical side of gastric mucosa with the ClC-2 agonist SPI-8811 (1 μmol/L) nullified decreases in TER due to acid injury (Figure 1A). Epithelial permeability was assessed by mucosal-to-serosal fluxes of H-mannitol in control and acid-injured tissues in the presence or absence of apical SPI-8811. In agreement with TER responses, acid-injured tissues exhibited increases in permeability to H-mannitol as compared to control uninjured tissue. Pretreatment of the apical surface of mucosal tissues SPI-8811 ablated changes in permeability noted in untreated acid-injured tissues (Figure 1B).
subsequently exposed to acid for 90 min had evidence of localization of occludin to the TJ.

Expression of occludin in gastric mucosal tissues
Since SPI-8811 prevented increases in paracellular permeability in response to acid, based on reduced \(^3\)H-mannitol fluxes and apical epithelial localization of occludin, we evaluated expression of occludin. Occludin expression was studied in detergent soluble and detergent insoluble gastric mucosal fractions, using-actin expression as a loading control. Tissues exposed to NR had expression of multiple bands clustered at 65 kD in the detergent insoluble fraction. The finding of several bands for occludin at its expected molecular weight has been attributed to multiple occludin phosphorylation states. The presence of occludin solely in the insoluble fraction was interpreted as an indication of this protein’s propensity to localize to the TJ. In contrast to tissues bathed in NR, there was very little expression of occludin in acid-treated tissues, possibly because acid injury was severe enough to cause surface epithelium and interepithelial TJs. On the other hand, occludin expression in the detergent insol-

Figure 2 Histological findings in acid-injured gastric mucosal tissues. A: Tissues bathed in normal Ringer’s appeared uninjured after 90 min in Ussing chambers, whereas those exposed to acid had substantial evidence of epithelial injury (× 100). Pretreatment with SPI-8811 ameliorated injury evident in acid-injured tissues. Each panel is representative of at least 3 separate animals; B: Periodic acid-schiff stain (PAS) staining findings in control tissues bathed in Ringer’s solution for 90 min appeared to have normal expression of surface mucus by PAS-alcian blue staining whereas tissues exposed to acid had distinct loss to surface mucus staining (× 100). However, pretreatment with SPI-8811 prevented the loss of mucus in response to acid and thus preserved the surface mucus; C: Immunofluorescence findings for tight junction protein occludin reveals control tissues bathed in Ringer’s solution for 90 min appeared to have normal immunolocalization of the tight junction protein occludin (green fluorescence) whereas tissues exposed to acid had very little visible apical epithelial occludin (× 200). However, pretreatment with SPI-8811 prevented the disorganized appearance of occludin in response to acid. Note the specific localization of occludin in each panel (arrows).
with SPI-8811, tissues were pretreated with pharmaco-
not shown). In more targeted studies in tissues pretreated
blocked the ability of SPI-8811 to reduce acid injury (data
basolateral) and SPI-8811 (1 μmol/L, apical), which
secretion in SPI-
and CFTR. Pretreatment of acid-injured mucosa with the
CIC-2 inhibitor ZnCl₂ (300 μmol/L, apical) abolished the
gastro protective properties of SPI-8811 as determined
by change in TER and mannitol fluxes. On the other
hand, pretreatment with CFTR inhibitor 172 (10 μmol/L,
apical) had no effect (Figure 4).

Expression of CIC-2 and CFTR in porcine gastric mucosa

The literature is not clear about the expression of CIC-2
and CFTR in the porcine gastric mucosa, aside from a few
studies that describe gastric mucosal changes in CFTR
mouse mutants[19]. Therefore, western analyses
were performed to study the expression of CIC-2 and

Role of Cl channels in SPI-8811-mediated protection of TER
and barrier permeability in acid-injured gastric mucosa

To explore the potential role of Cl secretion in SPI-
8811-associated gastro protection, mucosa was treated
with the NKCC1 inhibitor bumetanide (100 μmol/L,
basolateral) and SPI-8811 (1 μmol/L, apical), which
blocked the ability of SPI-8811 to reduce acid injury (data
not shown). In more targeted studies in tissues pretreated
with SPI-8811, tissues were pretreated with pharmacological inhibitors of the apical chloride channels CIC-2

Figure 3 Expression of tight junction proteins in membrane fractions in acid
injured porcine gastric mucosa. A: Western analysis revealed expression of occludin in the
detergent insoluble in tissues treated with normal Ringer’s (NR) alone,
with very little discernable occludin in the detergent soluble fraction. On the other
hand, treatment with acid (pH of 1.5 for 90 min) markedly reduced the expres-
sion of occludin in the detergent insoluble fraction, with a similar lack of occludin
expression in the detergent soluble fraction. Pretreatment of tissues with mucosal
SPI-8811 resulted in expression of occludin in the different fractions to a very similar
extent as control tissues; B: Western analysis revealed expression of whole occludin in
either control, acid injured and SPI-8811 pretreated and acid injured porcine
gastric mucosa from Ussing chambers. Acid injury markedly reduced expression of
whole occludin compared to control while pretreatment with SPI-8811 preserved the
loss of occludin from acid injury. DS: Detergent soluble; DIS: Detergent insoluble.

Figure 5 Expression of CIC-2 protein in porcine mucosa. Mucosal homogenates
from porcine stomach were studied for expression of CIC-2 by western
blotting. Expression of CIC-2 protein at its expected molecular weight (98 kD) was
evident in all lanes. However, there appears to be greater expression of tissues
bathed in Ringer’s solution as compared to those subjected to acid or acid and
SPI-8811 (n = 3). NR: Normal Ringer’s.

Figure 4 Electrical responses and 3H-mannitol fluxes of acid-injured porcine
gastric mucosa. A: Porcine gastric mucosa exposed to acid (pH 1.5 for
90 min) exhibited a significant drop in transepithelial electrical resistance (TER),
which was completely blocked by pretreatment with SPI-8811. In attempt to dis-
cern which chloride channel was involved in the gastro protective mechanism
of SPI-8811, CIC-2 and cystic fibrosis transmembrane conductance regulator
(CFTR) were inhibited. Addition of the CIC-2 inhibitor ZnCl₂ ameliorated the pro-
tective effect of SPI-8811 (TER, 100 Ω·cm² vs 80 Ω·cm²) whereas the CFTR
inhibitor CFTR inhibitor 172 had no effect (TER, 100 Ω·cm² vs 100 Ω·cm²). Val-
ues represent mean ± SE, n = 6; P < 0.05, P < 0.01 vs all treatment groups;
B: As an alternate measure of barrier permeability mucosal-to-serosal of 3H-
mannitol flux were performed, and revealed a significant increase in mannitol
permeability in acid injured tissues (P < 0.01). Pretreatment with SPI-8811
blocked the increase in 3H-mannitol flux caused by acid injury, whereas block-
ade of CIC-2 inhibitor ZnCl₂ block the protective effect of SPI-8811 (3H-mannitol flux, 0.04 μmol/L·cm² vs 0.12 μmol/L·cm², P < 0.05) on permeability but CFTR
inhibitor had no effect on the level of protective properties of SPI-8811 on per-
meability (3H-mannitol flux, 0.04 μmol/L·cm² vs 0.05 μmol/L·cm², P < 0.01). Values
represent mean ± SE, n = 6. NR: Normal Ringer’s.
CFTR in porcine gastric mucosa. As shown in Figure 5, CIC-2 was expressed approximately 98 kD in porcine gastric homogenates. CIC-2 expression was observed in porcine gastric mucosa which confirmed the expression of CIC-2 in porcine stomach (Figure 5). Western analyses showed no evidence of CFTR in gastric mucosa, whereas CFTR was clearly present in porcine jejunum as a positive control (data not shown).

**DISCUSSION**

Prior studies have provided evidence for a critical role of CIC-2 in the recovery of mucosal barrier function in ischemic-injured intestine[19,21,22,27,28]. For example, Lubiprostone has been shown to interact with CIC-2 channels and enhance repair of the epithelial barrier after acute injury by ischemia[29,30,31]. The aim of the present study was to determine if an alternate prostone, SPI-8811, had a protective role against mucosal injury, and we chose to investigate this in the stomach. Acid plays an integral role in gastric mucosal ulceration[3,6,8,32]. Considering the physiological presence of acid to aid digestion in the stomach, mechanisms to provide gastro protection against injury in predisposed patients are critical. To date, this has principally included agents that ameliorate acid secretion. However, according to the results of the present study, prostones appear to provide protection in the face of low levels of pH, which would allow for continued acid secretion for normal digestive processes. Acid causes injury to the gastric mucosa in part by disrupting the TJ protein complexes, thereby increasing epithelial permeability. This in turn allows further injury by permitting permeability to luminal acid, ultimately causing erosion and then ulceration as the mucosa becomes progressively disrupted[3,14,24,32]. Among the established methods to study gastro protection and acute gastric injury are the direct application of acid or ethanol to gastric mucosa[15,30]. The first aim of our study was to produce acute acid injury resembling ulceration in clinical patients in order to study gastro protection. Accordingly, acid Ringer’s solution was applied to the mucosal surface as previously described[15]. To mimic peptic ulcer disease, the body of the porcine stomach was used in this study. Optimum gastric injury was achieved (based on TER and histological examination) with a pH of 1.5, over a time period of 90 min. In each of the experiments, SPI-8811 was applied to the gastric mucosa prior to acid injury on the apical surface. Earlier studies have shown that Lubiprostone activates CI secretion by a mechanism associated with recruitment of the TJ occludin in porcine intestine, thereby aiding recovery of barrier function[1,6,22,34,35]. Gastric mucosa was pretreated with SPI-8811 prior to induction of acid injury and showed evidence of gastro protection as evidenced by blockage of changes in TER and mannitol fluxes in tissues exposed to acid. In addition, histological changes in response to acid were ameliorated by SPI-8811. Because of the important role of TJs in maintenance of barrier function, we also examined the localization of occludin in tissues pretreated with SPI-8811 and exposed to acid. Tissues injured by acid alone appeared to have complete loss of apical epithelial occludin, possibly because of loss of cells due to severe injury, whereas tissues pretreated with SPI-8811 had evidence of apical epithelial occludin similar to that of control tissues. Further studies evaluating detergent soluble and insoluble fractions using western analyses confirmed the loss of occludin in tissues treated with acid. The reason for the loss of occludin, rather than intracellular movement of occludin, is unknown. The simplest explanation would be that the extensive reduction in pH resulted in loss of TJ proteins into the lumen, followed by erosion of epithelium and ultimately ulceration. Tissues pretreated with SPI-8811 evaluated by immunofluorescence were protected from this effect, with localization of occludin to the apical epithelium similar to that of control tissues. Additionally, occludin remained in the detergent insoluble fraction in western analyses under the influence of SPI-8811 indicating localization to the TJs.

The next aim of the study was to investigate the role of CI channels in gastro protection, given that SPI-8811 is a purported CI secretagogue. Because CIC-2 has been shown to be localized to interepithelial TJs in intestinal studies[29], and in gastric mucosa in the parietal cells[29,30], is a target for the prostones, we were particularly interested in this channel. The CFTR served as an alternate possibility. Our data demonstrated that SPI-8811-mediated gastro protection against acid injury was attenuated by the CIC-2 inhibitor ZnCl: whereas the selective CFTR inhibitor 172 had no effect. Although CFTR is difficult to pharmacologically inhibit, our Western analyses showed a lack of CFTR expression in porcine gastric mucosa as compared to robust expression in jejunal mucosa. In previous studies, investigators have shown that CFTR knockout mice are vulnerable to gastric ulcers due to loss of bicarbonate secretion. The lack of CFTR in porcine gastric mucosa especially in the fundus of stomach might partially explain susceptibility to acid injury in the present studies[30]. Taken together, these studies suggest that activation of CIC-2 by SPI-8811 is integral to the mechanism of gastro protection. Further studies will be required to understand the mechanisms underlying these findings.

There are opposing views regarding the role of CIC-2 in gastric chloride secretion[29,36] suggested involvement of CIC-2 in gastric acid secretion while another group[38] indicated that CIC-2 chloride secretion plays no role in production of HCl. It is also noteworthy that the apical CIC-2 channel has been suggested to serve as a route for both bicarbonate and CI exit into the lumen[29,39]. This raises the possibility that CIC-2-mediated bicarbonate secretion attenuates acid injury to some extent by raising the pH. However in our studies, constant monitoring and adjustment of pH to a level of 1.5 would nullify this possibility. We also found that SPI-8811 produces a gastro protective effect not only via increasing gastric pH, but also by protecting and restoring the gastric mucus level (Figure 2B). The importance of gastro protection via mucus protection has been previously shown[7,40,34].
CIC-2 localizes to the TJ in the intestine\(^{21,27,28,41}\) and this localization facilitates the interactions with TJ proteins and associated regulatory molecules. For example, recent studies have shown that CIC-2 is required for rapid reassembly of TJ proteins after ischemic injury in murine intestine\(^{29}\). The rapid process of repair is a hallmark of gastric mucosal barrier function, and may involve rapid restoration of TJs during restitution. Although this study does not completely answer questions as to the mechanism of SPI-8811, it does provide convincing evidence for the ability of SPI-8811 to protect the gastric mucosa against acid, and it does suggest this activity is at least in part attributable to a mechanism related to CIC-2 regulation.

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**COMMENTS**

**Background**

Important gastric diseases such as peptic ulcer disease are aggravated by acid secretion. Injury caused by acid is characterized by damage to the gastric epithelium lining the gut. Mechanisms are in place to rapidly repair epithelial defects, and prevent epithelial barrier dysfunction and thus protect the gastric mucosa and further acid injury. Currently studies have shown the importance of the interepithelial tight junctions (TJs) in recovery of the epithelial barrier. Studies, particularly in the intestine, have shown particularly in that prostogenes increase the rate of epithelial recovery via re-assembly of TJs.

**Research frontiers**

The prostate lube prostone, a new medication on the market indicated for the treatment of chronic constipation and irritable bowel syndrome has its effect on the chloride channel CIC-2. This channel is unusual in that it is located within TJs. These channels are involved in secretion of chloride in the intestine, but until now very little was about the role of CIC-2 in the stomach. Recent studies have shown that CIC-2, when activated by prostones, play an important role in re-assembly of TJs, resulting in increased rate of epithelial repair in the intestine. This study was performed to see if a prostone (cobiprostone, SPI-8811) hastened repair in the gastric mucosa via a mechanism involving TJs.

**Innovations and breakthroughs**

Activation of one of the chloride channels, CIC-2, is an innovative way to induce reassembly of TJs during repair of the epithelial mucosal barrier. Gastric epithelium is continuously exposed to gastric acid and in the case of peptic ulcer disease the gastric acid secretion disrupts the barrier and thus aggravates the condition. In such cases, reducing acid secretion by using various acid secretory antagonists is the most common way to prevent gastric injury. Other alternatives to prevent breakdown of the gastric barrier function are limited. The present data show an innovative breakthrough in the treatment of acid induced gastric epithelium. The authors’ findings were consistent with prior studies showing enhanced repair with prostones in the intestine at the level of TJs.

**Applications**

By understanding the role of prostate activation of CIC-2 in acid induced gastric porcine mucosa, this study might represents a future strategy to therapeutic intervention in patients with non-steroidal anti-inflammatory drugs injury and peptic ulcer disease. Further basic science research followed by clinical trials will be needed to determine the validity of these findings.

**Terminology**

The term CIC-2 is used to describe a chloride channel in the gut epithelium that is localized to interepithelial TJs. The term prostate refers to a new group of compounds which are distinct from prostaglandins and specifically activate CIC-2.

**Peer review**

The present study was performed using porcine tissues; it demonstrates that the novel CIC-2 agonist SPI-8811 stimulates recovery of barrier function in acid injured porcine gastric mucosa by preventing loss of the TJ protein occludin. The data were appropriately analyzed and indicate SPI-8811 has an impact on protecting epithelial barrier function by preventing breakdown of TJs and thus prevent further deep injury. Additional molecular studies need to be performed to understand how CIC-2 interacts with prostones to protect the epithelial TJs.
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