Prostaglandin E₂ Produced by *Entamoeba histolytica* Signals via EP4 Receptor and Alters Claudin-4 to Increase Ion Permeability of Tight Junctions

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***Entamoeba histolytica* is a protozoan parasite that causes amebic dysentery characterized by severe watery diarrhea. Unfortunately, the parasitic factors involved in the pathogenesis of diarrhea are poorly defined. Prostaglandin E₂ (PGE₂) is a host lipid mediator associated with diarrheal diseases. Intriguingly, *E. histolytica* produces and secretes this inflammatory molecule. We investigated the mechanism whereby ameba-derived PGE₂ induces the onset of diarrhea by altering ion permeability of paracellular tight junctions (TJs) in colonic epithelia. PGE₂ decreased barrier integrity of TJs in a dose- and time-dependent manner, as measured by transepithelial resistance. PGE₂ signals were selectively transduced via the EP4 receptor. Furthermore, PGE₂ signaling decreased TJ integrity, as revealed by EP receptor-specific agonist and antagonist studies. Loss of mucosal barrier integrity corresponded with increased ion permeability across TJs. Subcellular fractionation and confocal microscopy studies highlighted a significant spatial alteration of an important TJ protein, claudin-4, that corresponded with increased sodium ion permeability through TJs toward the lumen. Moreover, PGE₂-induced luminal chloride secretion was a prerequisite for alterations at TJs. Thus, the gradient of NaCl created across epithelia could serve as a trigger for osmotic water flow that leads to diarrhea. Our results highlight a pathological role for *E. histolytica*-derived PGE₂ in the onset of diarrhea. (Am J Pathol 2011, 179:807–818; DOI: 10.1016/j.ajpath.2011.05.001)**

Entamoeba histolytica is an enteric-dwelling protozoan parasite that infects an estimated 500 million people worldwide.¹ Alarming, *E. histolytica* accounts for more than 100,000 deaths annually.² The disease amebiasis,

which manifests as amebic colitis in 10% to 20% of infected subjects, is characterized by acute watery and/or bloody diarrhea.^{1,3–5} The mechanism by which *E. histolytica* causes acute diarrhea is not clearly understood. *E. histolytica* in contact with colonic epithelial cells induces a robust inflammatory response involving proinflammatory cytokines and other inflammatory mediators that causes what is known as invasive or inflammatory diarrhea.⁶ However, the acute nature of the disease indicates the possibility of pathogenesis even in the absence of parasite-epithelial contact. We speculate that *E. histolytica* can cause diarrhea in an epithelial contact-independent manner; however, the parasite factor or factors responsible for this effect is not clearly understood.

Prostaglandin E₂ (PGE₂) is an important host inflammatory mediator that is associated with various diarrheal pathologies, including cholera, entero-invasive bacterial diseases, and inflammatory bowel diseases.^{7–10} PGE₂ levels are increased by 10-fold in the colonic mucosa during *E. histolytica* infection.¹¹ Amebic trophozoites induce host cells such as macrophages, polymorphonuclear cells, and colonic epithelial cells to synthesize PGE₂.^{11,12} Remarkably, *E. histolytica* trophozoites also constitutively produce and secrete PGE₂.¹³ The identity/similarity of the parasite PGE₂ with that of the host has been confirmed by gas chromatography/mass spectrometry analysis.¹⁴ Nevertheless, PGE₂ is the only prostanoid present in the parasite SPs. Parasitic production of PGE₂ was significantly increased in the presence of arachidonic acid (AA),^{13,15} the precursor for PGE₂, which is usually present in high concentrations in the gut.^{16,17} More importantly, *E. histolytica* has a COX-like gene that encodes a functional cyclooxygenase enzyme for the biosynthesis of PGE₂; however, *E. histolytica* COX is prim-

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itive, and is unique in its pharmacological sensitivity to nonsteroidal anti-inflammatory drugs in being sensitive only to aspirin (ASA).¹⁵ Ameba COX has little homology to COX-1/2 enzymes from different species, in that the AA-binding domain and heme-coordinating and catalytic sites are absent.¹⁵ At present, we do not know the exact functional/biological significance of ameba-derived PGE₂ in the pathogenesis of amebiasis. We have recently shown that *E. histolytica* PGE₂ signals via the host epithelial EP4 receptors to induce robust IL-8 secretion that could augment inflammation.¹⁸ Because PGE₂ is strongly associated with various diarrheal diseases, however, we reasoned that ameba-derived PGE₂ might play a major role in the onset of diarrhea during acute amebic colitis.

In general, secretory diarrhea is characterized by an alteration of the ion barrier of intestinal epithelium¹⁹ through increased luminal secretion of chloride ions (anions), a phenomenon that is observed with cholera toxin in the ileal mucosa.²⁰ To maintain charge balance in the lumen, sodium ions (cations) are pulled across the epithelium, also drawing water molecules and thus causing diarrhea.¹⁹ PGE₂ is a potent colonic epithelial chloride ion secretagogue^{21–23}; however, it is not known whether it induces sodium ion flux across the epithelium. Colonic epithelial tight junctions (TJs) act as an important barrier for paracellular movement of macromolecules and ions across the epithelium.^{24–26} Among the 40 different proteins that constitute the TJ complex, the claudin family is responsible for ion barrier maintenance of the paracellular space.^{27,28}

We speculate that PGE₂ secreted by *E. histolytica* breaks the TJ ion barrier by selectively altering claudin family members, allowing free paracellular permeability to sodium ions. Any alteration in the TJ ion barrier will markedly affect epithelial transport mechanisms associated with electrolyte and water imbalance. Here, we present a novel mechanism by which *E. histolytica* disrupts colonic TJ barrier function and offer important insight into the cellular and molecular basis of epithelial barrier/ion transport function in intestinal amebiasis. To date, there has been no report suggesting a contact-independent disruption of TJs by *E. histolytica*. The focus of our study was therefore to identify a role for *E. histolytica*-derived PGE₂ in altering the colonic epithelial TJ ion barrier and to define the mechanism by which PGE₂ causes diarrhea.

Materials and Methods

SPs of *E. histolytica*

Secreted products (SP) of *E. histolytica* were prepared and collected as described previously.²⁹ Briefly, trophozoites of HM1-IMSS, a highly virulent strain of *E. histolytica*, were cultured until log phase in TYI-S-33 medium containing 20% serum. Trophozoites were washed three times in ice-cold Hanks' balanced salt solution (HBSS), adjusted to a final concentration of 1×10^7 /mL HBSS, and incubated for 2 hours at 37°C under periodic swirling. The resulting SP were collected by centrifugation ($1000 \times g$ for 5 minutes) and assayed for protein amount

using Bradford's reagent, normalized (0.1 μ g/ μ L of SP), aliquoted, and stored at -80°C until further use. Trophozoite viability after incubation was $>95\%$ as determined by trypan blue exclusion assay. In some experiments, *E. histolytica* trophozoites were incubated with a nonspecific COX-1/2 inhibitor (1 mmol/L ASA) or with a specific COX-1/2 inhibitor (50 μ mol/L indomethacin) for 16 hours. COX inhibitors were removed by centrifugation, followed by 2 hours of incubation in fresh HBSS for collecting SP as described previously.¹⁸ In another experiment, 100 μ mol/L AA was added to trophozoites in HBSS at the start of the 2-hour incubation and SP was collected.

Quantification of PGE₂ in SPs

PGE₂ in SP was assayed using an Assay Designs Correlate-CLIA kit (no. 910–001; Enzo Life Sciences, Plymouth Meeting, PA). This highly sensitive PGE₂ chemiluminescence enzyme immunoassay is designed for quantitative determination of human PGE₂ in biological samples with $<0.1\%$ cross-reactivity with AA. The assay was performed according to the manufacturer's protocol.

Colonic Cells and Reagents

For all experiments, T84 human colonic cells (ATCC, Manassas, VA) were used, maintained in Dulbecco's modified Eagle's medium with Ham's F-12 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μ g/mL streptomycin sulfate and 20 mmol/L HEPES (Sigma-Aldrich, St. Louis, MO). In one experiment, Caco-2 cell line cells were used, maintained in modified Eagle's medium with 10% fetal bovine serum, 100 units/mL penicillin, 100 μ g/mL streptomycin sulfate, and 20 mmol/L HEPES (Sigma-Aldrich). PGE₂, EP receptor-specific agonists, and antagonists were obtained from Cayman Chemical (Ann Arbor, MI), unless otherwise indicated. Antibodies for TJ proteins, F-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Invitrogen (Carlsbad, CA), Abcam (Cambridge, MA), and Calbiochem (San Diego, CA), respectively; all other chemicals were procured from Sigma-Aldrich.

Transwell Preparation

T84 cells were incubated at 37°C in 5% CO₂ and passaged when the monolayer reached 90% confluency. Cells between passage numbers 64 and 68 were used for plating on culture plates for Transwell studies. To passage cells, the monolayer in a T75 flask was rinsed with 5 mL of sterile Dulbecco's PBS and incubated with 1 mL of trypsin-EDTA for 8 to 10 minutes. Detached cells were resuspended in medium to a final concentration of 1×10^6 cells/mL and the medium was mixed vigorously to avoid clumps. To prepare a 12-well Transwell plate for electrophysiological studies, 5×10^4 cells were seeded on the apical side of the membrane containing 500 μ L medium; the basolateral side was bathed with 1.5 mL of medium alone. Transwell plates were fed the day after seeding and every alternate day thereafter and were used for experiments in approximately 8 to

10 days. For TJ protein analysis and immunofluorescence studies, T84 cells plated on 6-well plates/coverslips were used.

Measurement of TER

T84 cells grown to 100% confluence on polyethylene membrane inserts (12-mm diameter, 0.4- μ m pore size) (catalog no. 3460, Corning Costar Transwell; Corning Life Sciences, Corning, NY) were used. Transepithelial resistance (TER) was measured using a Millicell-ERS apparatus (Millipore, Bedford, MA). When a stable resistance of $>2000 \Omega \cdot \text{cm}^2$ was reached, the medium from either the apical or basal compartment was replaced with medium containing the desired stimulant and TER was measured. Changes in TER in response to stimuli were normalized to that of the baseline resistance (resistance at the starting time point) and represented as percentage of the control over a desired time course. For experiments involving EP receptor agonists/antagonists, the concentration and the conditions for treatment/pretreatment were determined based on previous reports.^{30–34}

Paracellular Permeability Study

The permeability of TJs to macromolecules/solute in response to PGE₂ was measured by studying the apical to basal translocation of ¹⁴C-labeled ethanolamine (a small paracellular tracer, $\sim 4.9 \text{ \AA}$ in diameter), as described previously.³⁵ The tracer was used at a concentration of 0.33 $\mu\text{Ci}/\text{well}$. After incubation with tracer for the desired time intervals, 40- μL samples from both chambers were collected in separate scintillation vials containing 10 mL of scintillation fluid. Radioactivity was measured using a Beckman liquid scintillation counter (Beckman Coulter, Brea, CA). Permeability to [¹⁴C]ethanolamine was measured as the apparent permeability coefficient P_{app} and was expressed as the transport enhancement ratio R , as described previously.³⁶

NaCl Dilution Potential Assay and Measurement of Sodium Potential

A 20% NaCl dilution potential assay was performed as described previously.³⁷ Briefly, medium was discarded from a Transwell plate with a confluent T84 monolayer and cells were bathed in PBS + bovine serum albumin (BSA) (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 2 mmol/L KH₂PO₄, and 0.6% BSA), with 0.5 and 1.5 mL in the apical and basal chambers, respectively. After apical stimulation with 1 $\mu\text{mol}/\text{L}$ PGE₂ in PBS + BSA, 20% dilution in either the apical or the basal chamber was developed by replacing 100 or 300 μL of apical or basal solution with a solution of equally osmolar mannitol + BSA (140 mmol/L mannitol, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 2 mmol/L KH₂PO₄ and 0.6% BSA), respectively. The electrical potential was recorded before and after 20% dilution and at 5-minute intervals from the start until 30 minutes. The 20% dilution potential difference was calculated as the difference between the initial

potential and the potential after dilution. For measuring sodium potential, T84 monolayer on a Transwell plate was washed with PBS + BSA and stimulated apically with 1 $\mu\text{mol}/\text{L}$ PGE₂ in mannitol + BSA. At various time points, 25- μL samples were collected from the apical chamber and sodium potential was measured using a highly sensitive sodium electrode (Thermo Orion, Beverly, MA), as described previously.³⁵ For experiments involving pharmacological inhibition of membrane sodium channel/transporters or cystic fibrosis transmembrane regulator (CFTR), inhibitor concentration and conditions for pretreatment were determined based on previous reports.^{38–40}

Cell Fractionation and Western Blotting for TJ Proteins

T84 cells grown to confluence on regular 6-well plates were fractionated after appropriate treatment, based on a previously described protocol⁴¹ with slight modifications. The cell monolayer was washed twice with ice-cold PBS, scraped using a rubber policeman, and collected in an Eppendorf tube. Cells were centrifuged at $1250 \times g$ for 5 minutes at 4°C. The pellet was resuspended in 400 μL of ice cold cell lysis buffer (50 mmol/L Tris-HCl, 140 mmol/L EDTA, 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride and protease inhibitor cocktail tablet) containing 1% Triton X-100, incubated for 30 minutes on ice and centrifuged at $20,000 \times g$ for 30 minutes at 4°C. The supernatant was labeled as Triton X-100 soluble fraction (SF) or noncytoskeletal fraction. The pellet was again resuspended in 200 μL of cell lysis buffer containing 1% SDS, and then was sonicated and centrifuged at $20,000 \times g$ for 30 minutes at 4°C. The resulting supernatant was labeled as Triton X-100 insoluble fraction (IF) or cytoskeletal fraction. Protein content of subcellular fractions was estimated using the Bradford method and was adjusted for a final concentration of 0.5 $\mu\text{g}/\mu\text{L}$. Equal volume of 1 $\times$ sample buffer was added to the samples, boiled at 100°C for 10 minutes, and used for Western blotting. Approximately 2.5 to 5.0 μg of total protein was loaded per well of 7.5% to 15% SDS-PAGE (depending on the molecular weight of the TJ protein being probed) and electrophoresis was performed. Proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) followed by blocking in 5% skim milk powder in TBS-T (20 mmol/L Tris-HCl, pH 7.5, 500 mmol/L NaCl, 0.1% Tween 20) for 1 hour at room temperature. Membranes were incubated with appropriate primary antibodies in 1% skim milk-TBS-T at 4°C, overnight. Blots were washed three times with TBS-T and then incubated in horseradish peroxidase-conjugated secondary antibodies in 1% skim milk-TBS-T for 2 hours at room temperature. The blots were washed with TBS-T and were developed using Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA) according to the manufacturer's instructions.

Figure 1. Effect of secreted components of *E. histolytica* on TER of T84 monolayers. T84 monolayers on Transwell plates that reached a stable TER were exposed on the apical side to various amounts (8, 24, 48 µg) of Wt ameba SP (Wt-SP). SP was collected from 1.5×10^7 trophozoites in 1.5 mL of HBSS and protein concentration normalized to 0.1 µg/µL. TER was measured over 5, 30, and 60 minutes and the mean value was expressed as percentage of the control and compared with that of the control. The mean baseline TER of untreated monolayers at the start of experiments was $2744 \pm 516 \Omega \cdot \text{cm}^2$. * $P < 0.05$; *** $P < 0.001$.

Confocal Immunofluorescence Microscopy

T84 monolayers grown on glass coverslips in regular six-well culture plates were used. After appropriate treatment, monolayer was washed with ice cold PBS and fixed with 3.75% paraformaldehyde at 4°C for 30 minutes. Fixed cells were permeabilized using 0.5% Triton X-100 in PBS for 15 minutes and blocked with 5% BSA in PBS-0.5% Tween 20 (PBS-T) for 1 hour at room temperature. Monolayers were incubated overnight in 1:100 dilution of appropriate primary antibodies at 4°C. After two washes in PBS-T, monolayers were coincubated with fluorescein isothiocyanate or phycoerythrin-conjugated secondary antibodies (1:200 dilution) for 1 hour at room temperature. Coverslip was mounted on clean glass slide using Vectashield (Vector Laboratories, Burlingame, CA). Slides were examined using a FluoView FV1000 confocal immunofluorescence microscope (Olympus). Immunofluorescence signals from the tags for respective proteins were determined in individual *en face* (*xy* axes) planes throughout the cellular *z* axis at 0.35-µm intervals. Z-planes obtained were stacked and analyzed using Volocity software version 5.4 (PerkinElmer, Waltham, MA) to obtain P_{xz} and three-dimensional (3D) reconstructions of the Z-stacks.

Statistical Analysis

Data were analyzed by two-way analysis of variance followed by a Bonferroni post hoc test for comparison between groups using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). In the figures, data are reported as means $\pm$ SD of three independent experiments.

Results

Secreted Components of *E. histolytica* Alter TER of Colonic Monolayer

To test the hypothesis that secreted components derived from highly virulent wild-type (Wt) *E. histolytica* alter the

integrity of the colonic epithelial monolayer during pathogenesis of intestinal amebiasis, the effect of SP on TER of T84 colonic epithelial monolayer was analyzed. When the monolayer was exposed on the apical/luminal side to SP, there was a decrease in the integrity of TER that occurred in a dose- and time-dependent manner, compared with the nonstimulated controls (Figure 1). With high doses of SP (48 µg), there was a 55% decrease in TER as early as 5 minutes, followed by a gradual recovery but with a 28% decrease ($P < 0.001$) remaining even at 60 minutes. This is in contrast to the response observed with low doses of SP (8 µg), which caused a modest 19% decrease in TER at 5 minutes ($P < 0.05$), followed by recovery to control values as early as 30 minutes. An intermediate dose of SP (24 µg) caused 34% ($P < 0.001$) and 19% ($P < 0.05$) decreases at 5 and 30 minutes, respectively, with recovery to control values at 60 minutes. Thus, the effects of *E. histolytica* SP on TER follow a dose-response relationship. More importantly, these results show that alteration in colonic epithelial barrier function occurred in the absence of parasite-epithelial cell contact.

PGE₂ Secreted by *E. histolytica* Decreases Colonic TER

We have previously shown that trophozoites of *E. histolytica* constitutively produce and secrete PGE₂ and have confirmed its identity with mammalian PGE₂ by gas chromatography/mass spectrometry analysis.^{13–15} For the present study, we quantified PGE₂ release by live trophozoites under the same conditions as used to isolate SP in the previous experiments. PGE₂ was constitutively released and present in SP from Wt amebas (635.1 ± 15.67 pg/1.5 $\times 10^7$ trophozoites) (Figure 2). More importantly, when live trophozoites were incubated in the presence of AA substrate (AA-SP), there was a 754% increase in PGE₂ production. In previous studies,^{15,18} we have shown that among the known COX inhibitors studied only ASA (1 mmol/L) significantly inhibited the cell-free as well as the live *E. histolytica* COX-like enzyme. Accordingly, in the present study, 16-hour pretreatment of trophozoites

Figure 2. Quantification of PGE₂ produced by *E. histolytica*. SP collected from 1.5×10^7 trophozoites in 1.5 mL of HBSS were quantified for PGE₂ using a Correlate CLIA kit. AA-SP, SP collected from amebas incubated with 100 µmol/L AA for 2 hours; ASA 16 hours-SP and INDO 16 hours-SP, SPs collected from amebas pretreated for 16 hours with 1 mmol/L ASA or 50 µmol/L indomethacin, respectively; Wt-SP, SP from Wt amebas. *** $P < 0.001$ versus Wt-SP.

Figure 3. Effect of PGE₂ secreted by *E. histolytica* on TER of T84 monolayer. T84 monolayers that reached a stable TER of >2000 $\Omega \cdot \text{cm}^2$ were exposed on the apical side to 48 μg SP collected from either *E. histolytica* pretreated with ASA for 16 hours (ASA 16 hours-SP) or from nontreated Wt controls (Wt-SP). TER was measured over 5, 30, and 60 minutes and the mean values were compared with Wt-SP. * $P < 0.05$; ** $P < 0.01$.

with 1 mmol/L ASA inhibited PGE₂ production by 32% ($P < 0.001$), whereas indomethacin had no inhibitory effect. These data clearly show that live *E. histolytica* not only constitutively synthesize PGE₂ but that this synthesis is significantly inhibited by ASA and is markedly increased in the presence of AA substrate.

To determine whether PGE₂ produced by *E. histolytica* was the putative agonist present in SP that altered TER, the effect of SP (48 μg) derived from *E. histolytica* pretreated with ASA for 16 hours (ASA 16 hours-SP) was compared with that of untreated Wt control (Wt-SP). ASA 16 hours-SP significantly prevented the decrease in TER at all time points measured (Figure 3). These data confirm a critical role for PGE₂ produced by *E. histolytica* in altering TER. Because inhibition of COX by ASA was not 100%, we determined the contribution of other major putative virulence components secreted by amebas in altering TER. Experiments were done with a commercially available analog of serotonin (5-hydroxytryptophan), which is a known SP component, and with SP collected from amebas lacking both amebapore and cysteine proteinase 5 (CP5). Wt-SP treated with the CP5 inhibitor E-64 was also used. None of the virulence factors that can be found in SP significantly modulated TER (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Moreover, the inactive PGE₂ analog 15-keto PGE₂ did not decrease TER, confirming specificity for PGE₂.

Dose-Response Study Highlights a Differential Role for PGE₂ in Altering TER

Given that bioactive *E. histolytica*-derived PGE₂ is difficult to purify, and given that it is identical to mammalian PGE₂, all subsequent experiments were done with commercially available purified PGE₂. This strategy also eliminated any confounding effects of other ameba molecules that could affect TER function. Having identified a dose-response relationship for SP in altering TER (Figure 1), we determined whether purified PGE₂ could also mimic the same effect. More importantly, we analyzed a dose-response of PGE₂, considering the fact that PGE₂ can exert diverse biological functions based on the amount re-

leased around target cells.⁴² Various doses of purified PGE₂ had a differential effect on TER (Figure 4). A low dose of 8 nmol/L PGE₂ did not significantly alter TER. At higher concentrations, there was a precipitous decrease in TER as early as 5 minutes. At the highest concentration tested (1 $\mu\text{mol/L}$), there was a 70% decrease in TER as early as 5 minutes ($P < 0.001$). At an intermediate dose of 100 nmol/L there was a temporal fluctuation, but TER remained well below that of control. For all doses tested, TER tended to be restored to normal levels by 12 hours (720 minutes). These results highlight the importance of a concentration-dependent differential effect of PGE₂ in altering TER of the colonic epithelium. Moreover, a PGE₂-induced decrease in TER was noted in a different colonic epithelial cell line, Caco-2, demonstrating that the effect is not cell line-specific (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). Nonetheless, the magnitude of PGE₂-induced alterations in TER varied between T84 and Caco-2 cells. This discrepancy could be due to a difference in the baseline TER between these cell lines ($\sim 2700 \Omega \cdot \text{cm}^2$ for T84, compared with $\sim 1100 \Omega \cdot \text{cm}^2$ for Caco-2).

High-Dose PGE₂ Signals via EP4 Receptors to Decrease TER

PGE₂ binds and signals through four different subtypes of EP receptors: EP1, EP2, EP3, and EP4.⁴² To elucidate the EP receptor subtype specificity of PGE₂ for decreasing TER, we used a panel of EP receptor subtype-specific agonists: 17 phenyl trinor PGE₂ for EP1/3, butaprost for EP2, sulprostone for EP3, and PGE₁OH for EP4/2. Only the EP4 receptor agonist PGE₁OH caused a rapid decrease in TER (71% at 5 minutes, $P < 0.001$) (Figure 5A). The EP2 receptor agonist butaprost exerted a modest decrease in TER (21%) at 5 minutes, but recovered shortly thereafter and was then identical to the control. Notably, the effect of the EP4 receptor agonist was similar to that observed with high-dose PGE₂ (Figure 4). Specificity for signaling via the EP4 receptor was confirmed with a highly specific EP4 receptor agonist, ONO-AE1329, which caused a rapid decrease in TER (60% at 5 minutes, $P < 0.001$) and also with an EP4 receptor-

Figure 4. Dose-response of purified PGE₂ on TER. T84 monolayers that reached a stable TER of >2000 $\Omega \cdot \text{cm}^2$ were exposed on the apical side to various concentrations (8 nmol/L to 1 $\mu\text{mol/L}$) of commercially available purified PGE₂. The mean values of each group for 5, 30, 60, and 720 minutes were expressed as percentage of the control and were statistically compared with controls. ** $P < 0.01$; *** $P < 0.001$.

specific antagonist, L161982, which completely abrogated the effect of 1 $\mu\text{mol/L}$ PGE₂ (Figure 5B).

PGE₂ Secreted by *E. histolytica* Preferentially Signals via EP4 Receptor to Alter TER

To identify whether ameba PGE₂ preferentially signaled via the EP4 receptor to alter TER, T84 monolayers were pretreated for 6 hours with the EP4 receptor-specific antagonist L161982 and then were exposed to high concentrations (48 μg) of Wt ameba SP. As predicted, L161982 significantly attenuated the decrease in TER caused by ameba SP ($P < 0.01$ at 5 minutes; $P < 0.05$ at 30 minutes) (Figure 6). In contrast, the EP2 receptor-specific antagonist AH6809 did not inhibit the barrier-compromising effect of SP. These data clearly demonstrate that PGE₂ present in SP of *E. histolytica* was responsible for decreasing TER by selectively activating the EP4 receptor on colonic epithelia. Moreover, these results also confirm the specificity of ameba-derived PGE₂ in altering colonic TER. Although L161982 significantly prevented SP-induced decrease in TER, it could not completely abrogate the effect of SP, indicating that other ameba-derived factors can play a role in altering TER.

High-Dose PGE₂ Alters Charge but Not Size Selectivity of TJs

TER is the gold standard for the measurement of TJ integrity of contiguous epithelial monolayers. In fact, TJs act as dynamic barriers that selectively allow macromol-

Figure 5. PGE₂ signaling via EP4 receptor decreases TER. T84 monolayers on Transwell plates with stable TER (mean, $2422 \pm 262 \Omega \cdot \text{cm}^2$) were used. The mean values at 5, 30, and 60 minutes were expressed as percentage of control and compared statistically with that of control. **A:** The monolayers were treated separately with various EP receptor agonists (3 $\mu\text{mol/L}$ 17 phenyl trinor PGE₂, an EP1/3 agonist; 3 $\mu\text{mol/L}$ sulprostone, an EP3 agonist; 10 $\mu\text{mol/L}$ butaprost, an EP2 agonist; and 500 nmol/L PGE₁-OH, an EP4/2 agonist) and TER was recorded. **B:** The monolayers were exposed to 1 $\mu\text{mol/L}$ ONO-AE1-329, a highly specific EP4 receptor agonist. They were also exposed to 1 $\mu\text{mol/L}$ PGE₂ after 6 hours pretreatment with 10 $\mu\text{mol/L}$ of the EP4 receptor antagonist L161982. * $P < 0.05$; *** $P < 0.001$.

Figure 6. PGE₂ secreted by *E. histolytica* preferentially signals via EP4 receptor to alter TER. T84 monolayers with stable TER of $>2000 \Omega \cdot \text{cm}^2$ were pretreated individually with 10 $\mu\text{mol/L}$ of the EP4 receptor antagonist L161982 for 6 hours and with 10 $\mu\text{mol/L}$ of the EP2 receptor antagonist AH6809 for 45 minutes and then were exposed to 48 μg SP from Wt *E. histolytica*. The difference in mean TER value at 5, 30, and 60 minutes was compared with that of Wt-SP. * $P < 0.05$; ** $P < 0.01$.

ecules or ions to permeate the paracellular space. Thus, to determine whether the decrease in TER induced by high-dose PGE₂ corresponded with permeation of macromolecules across TJs, a paracellular tracer study using the lowest molecular weight tracer [¹⁴C]ethanolamine was performed. The P_{app} ratio (PGE₂ treated versus control) did not differ significantly, indicating that the TJ barrier was not altered for permeability to macromolecules (Figure 7A); however, a 20% NaCl dilution potential as-

Figure 7. Characterization of the effect of high-dose PGE₂ on paracellular permeability. **A:** Paracellular tracer study. Confluent T84 monolayers on Transwell plates that reached a TER of $>2000 \Omega \cdot \text{cm}^2$ were treated apically with or without 1 $\mu\text{mol/L}$ PGE₂ in medium containing the paracellular tracer [¹⁴C]ethanolamine (0.33 $\mu\text{Ci/mL}$). At 0.5, 3, and 12 hours, radioactivity in both chambers was measured and apical to basal translocation of the tracer, calculated as the apparent permeability coefficient (P_{app}) and expressed as transport enhancement ratio R (P_{app} PGE₂ treated versus P_{app} control). **B:** 20% NaCl dilution potential assay. After exposure to 1 $\mu\text{mol/L}$ PGE₂, 20% of apical/basal NaCl was replaced with an equal volume of equimolar mannitol. Potential difference was measured using a Millicell ERS apparatus every 5 minutes from 5 to 30 minutes and the dilution potential difference was compared with that of the control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

say, which tracks ion permeation across TJs, indicated that the TJ ion barrier was altered (Figure 7B). In fact, when 20% NaCl dilution was made on the apical side, the difference in dilution potential was significantly altered in the PGE₂-treated group, compared with that of controls ($P < 0.05$ at 15 minutes; $P < 0.001$ at 30 minutes), indicating basal to apical ion flow across the TJ. Notably, alteration in dilution potential was not symmetrical, as evident from a 20% NaCl dilution made on the basal side. Indeed, the dilution potential difference was not significant at any measured time point, which rules out apical to basal ion flow. These results clearly demonstrate a uni-directional (basal to apical) flow/flux of ions across paracellular TJs in response to high-dose PGE₂.

PGE₂ Increases Paracellular TJ Sodium Ion Permeability

As evident from our studies conducted by creating a NaCl gradient across epithelial monolayer, it is clear that either sodium (cation) or chloride (anion) passes through the TJ. Given the selectivity of TJs for cations,³⁵ we examined if there was an increase in the basal to apical, paracellular flow of sodium ions in response to high-dose PGE₂. The 1 μ mol/L PGE₂ treatment caused an increase in sodium potential on the apical side, with significance noted at 15 minutes, compared with control (increase of 18.63 mV, $P < 0.001$) (Figure 8A). To rule out the trans-

cellular release of sodium ions, however, important membrane sodium channels/transporters such as epithelial Na⁺ channels, the Na⁺K⁺2Cl⁻ cotransporter, and the NaCl cotransporter were pharmacologically inhibited using amiloride, bumetanide, and hydrochlorothiazide, respectively, and the effect of their inhibition on 1 μ mol/L PGE₂-induced decrease in TER was analyzed (Figure 8B). Inhibition of these channels/transporters did not prevent PGE₂-induced decrease of TER, which indicates a lesser role for membrane sodium channel/transporters in the apical release of sodium ions. These data indicate that the high-dose PGE₂-induced apical flux of sodium ions goes essentially through the TJ.

Activation of CFTR Is Essential for PGE₂-Induced Alterations in TER

PGE₂ is well known for activating apical membrane chloride channels that increase luminal secretion of chloride ions. In fact, apical stimulation of T84 monolayers caused a robust increase in potential difference, indicating luminal chloride secretion (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). To identify whether crosstalk occurs between PGE₂-induced secretion of chloride ions (increase in potential difference) and a decrease in TER (alteration at the TJ), the activity of CFTR, which is an abundantly expressed colonic epithelial chloride channel, was inhibited with glibenclamide, and the effect of that inhibition on high-dose PGE₂-induced decrease in TER was analyzed (Figure 8B). Of note, inactivation of CFTR significantly prevented the rapid decrease in TER, suggesting crosstalk between apical chloride secretion and alteration at the TJ.

High-Dose PGE₂ Shifts Claudin-4 from Cell Membrane to Cytoskeleton

TJ dynamics are regulated by a group of proteins that constitute the TJ complex. To correlate rapid changes in TER induced by PGE₂ with changes in TER due to structural alterations in TJs, we used a cellular fractionation technique that enables detection of TJ proteins that either dissociate from or remain integrated with the membrane complex. T84 monolayers treated with 1 μ mol/L PGE₂ for 5 minutes were fractionated into Triton-X 100 SF or IF. PGE₂-untreated monolayers were similarly processed in parallel. Validity of the subcellular fractions was confirmed by probing for the localization of subcellular markers (Figure 9). GAPDH was used as a cytoplasmic marker, F-actin as a cytoskeletal marker, and NaKAT-Pase as a membrane marker. GAPDH and NaKAT-Pase were localized in the SF and F-actin was detected in the IF. We assumed that any membrane-bound TJ proteins not linked to the actin cytoskeleton would fractionate in the SF, and that those linked to actin, even if present on the membrane, would segregate to the IF. Based on this assumption, we examined the subcellular status of the major TJ proteins, with special emphasis on claudins, because they play a critical role in regulating paracellular ion transport.^{27,28} There was a significant shift in clau-

Figure 8. Role of TJs in sodium ion permeability. **A:** Assessment of sodium ion flux. After treatment with or without 1 μ mol/L PGE₂, 20 μ L of apical medium was collected and examined for Na⁺ potential using the Orion Na⁺-sensitive electrode at 0, 5, 15, and 30 minutes, compared with control. **B:** The monolayers that reached a mean baseline TER of $3980 \pm 458 \Omega \cdot \text{cm}^2$ were individually pretreated for 45 minutes with 100 μ mol/L each of amiloride (a Na⁺ channel inhibitor) and hydrochloride thiazide (a NaCl cotransporter inhibitor), apically, and with 10 μ mol/L bumetanide (a Na⁺K⁺2Cl⁻ cotransporter inhibitor), basolaterally. Apical membrane chloride channels (CFTR) were inhibited by 6 minutes pretreatment on the apical side with 1 mmol/L glibenclamide. TER was measured at 5, 30, and 60 minutes after 1 μ mol/L PGE₂ exposure and was compared with that of the group treated with 1 μ mol/L PGE₂ alone. *** $P < 0.001$.

Figure 9. Subcellular fractionation studies highlighting PGE₂-induced cytoskeletal shift of claudin-4. **A:** After 5 minutes treatment with 1 μ M/L PGE₂, T84 cells were fractionated as Triton-X 100 soluble/noncytoskeletal fraction (SF) and Triton-X 100 insoluble/cytoskeletal fraction (IF) and were screened by Western blotting to detect localization of subcellular markers and analyzed for shift in six TJ proteins. **B:** Corresponding densitometry for the Western blots for the TJ proteins.

din-4 from the noncytoskeletal (SF) to cytoskeletal (IF) fraction in response to 1 μ M/L PGE₂, whereas the other TJ proteins tested were unaltered (Figure 9).

To visualize the nature of the spatial alteration in claudin-4, immunofluorescence confocal microscopy was performed (Figure 10A). T84 monolayers treated with 1 μ M/L PGE₂ for 5 minutes were probed for claudin-4 and ZO-1 (a marker for TJs), and the Z-stacks obtained were visualized for claudin-4 localization. Untreated T84 monolayers were used as controls. As seen in control monolayer, claudin-4 was predominantly present at TJs and a weak cytoplasmic localization of this protein was also observed. More importantly, claudin-4 was colocalized with ZO-1. However, in response to PGE₂, claudin-4 markedly dissociated from the TJ, and the redistribution of claudin-4 followed a mosaic-like pattern. The 3D reconstruction of Z-stacks confirmed that PGE₂ induced the dissociation of claudin-4 from the TJ (Figure 10B).

PGE₂ Secreted by *E. histolytica* Dissociates Claudin-4 from TJs

To determine specificity for PGE₂ secreted by *E. histolytica* in inducing spatial alteration of claudin-4, T84 monolayers were treated for 5 minutes with 48 μ g SP derived from either Wt amebas or from amebas pre-treated for 16 hours with ASA. Monolayers were then examined for claudin-4 localization as determined by confocal microscopy. SP dissociated claudin-4 from the TJ (with ZO-1 used as a marker for TJs); however, redistribution of claudin-4 followed a punctate-like pattern (Figure 11). As predicted, TJ integrity of claudin-4 was partially altered in T84 monolayers exposed to SP derived from trophozoites treated with ASA to inhibit PGE₂ production (Figure 11, A and B). Curiously, however, noticeable punctate-like patterns were still observed. Nonetheless, the chicken-wire-like TJ appearance of claudin-4 can be clearly seen in the ASA-SP treated panel and in the 3D reconstruction of Z-stacks (Figure 11B). These data demonstrate that PGE₂ secreted by *E. histolytica* can selectively dissociate claudin-4 from the TJ complex.

Figure 10. Confocal microscopic studies indicating high-dose PGE₂-induced dissociation of claudin-4 from TJs. T84 monolayers treated with or without 1 μ M/L PGE₂ for 5 minutes were probed for cellular localization of claudin-4. Claudin-4 is indicated by green fluorescence tag and ZO-1 (a marker for TJs) by red fluorescence. **A:** Z-stacks of slices along *xy* planes throughout the cellular *z* axis at 0.35- μ m intervals, as well as P_{xz}. In merged images, the blue arrow indicates sites where claudin-4 localizes with TJs and the white arrow points to dissociation from the TJ. Scale bar = 26.0 μ m. **B:** 3D reconstruction of Z-stacks for the same experiments. Scale: 1 grid unit = 5.30951 μ m.

Figure 11. PGE₂ secreted by *E. histolytica* dissociates claudin-4 from TJs. T84 monolayers treated or not for 5 minutes with 48 μg SP either from Wt amebas (Wt-SP) or from amebas pretreated for 16 hours with aspirin (ASA 16 hours-SP) were checked for localization of claudin-4 (green). ZO-1 was used as a marker for TJs (red). The red and green images were then merged. **A:** Z-stacks of slices along *xy* planes throughout the cellular *z* axis at 0.35-μm intervals. Scale bar = 26.0 μm). **Blue arrows** indicate colocalization and **white arrows** point to dissociation of claudin-4 from the TJ. **B:** 3D reconstruction of Z-stacks for the same experiments. Scale: 1 grid unit = 5.30951 μm.

Discussion

Intestinal amebiasis typically manifests as watery and/or bloody diarrhea. However, the parasite-associated factors and the mechanisms by which *E. histolytica* causes diarrhea have not been elucidated in detail. In the present study, we identified PGE₂ secreted by *E. histolytica* as one of the major factors that alter the colonic epithelial barrier and transport functions. We also highlight a role for PGE₂ in generating a NaCl gradient across epithelia that acts as a potential trigger for osmotic water flow across the paracellular space. Thus, we have unraveled a pathological role for *E. histolytica*-derived PGE₂ in the onset of watery diarrhea, the most common manifestation

of the intestinal amebiasis that occurs in 50 million cases annually during pathogenesis of amebic colitis. This is the first report identifying ameba-derived PGE₂ as a factor that triggers events potentially leading to diarrhea in diseased subjects.

Previous studies suggest that a direct contact between *E. histolytica* and the host epithelium is required for increased permeability of the colonic mucosa^{43,44} and hemorrhage, the causal events of inflammatory/bloody diarrhea. The present findings show that *E. histolytica* is capable of initiating disease pathogenesis even in a contact-independent manner. Recent studies have also highlighted the significance of contact-independent mechanisms in the pathogenesis of amebiasis. For example, CP5 secreted by *E. histolytica* has been shown to degrade colonic MUC2 mucin^{45,46} and to alter innate host defense, thus setting the stage for amebic invasion of the mucosa. Similarly, the roles of other SP components, such as serpins (serine proteinase inhibitors), that manipulate the host pathophysiological function have been identified.⁴⁷ Among the components secreted by *E. histolytica*, PGE₂ assumes greater significance, because it is a well-known lipid mediator produced by the host and is associated with various physiological and pathophysiological functions of the gastrointestinal tract.⁴² In particular, the pathological association of host-derived PGE₂ with various diarrheal diseases is well documented.^{7–9}

Notably, *E. histolytica* is the only known gut pathogen that synthesizes and secretes PGE₂,¹⁵ and the identity of the SP with host PGE₂ was confirmed by gas chromatography/mass spectrometry analysis.¹⁴ In the present study, using a highly sensitive CLIA kit designed to assay host PGE₂ in biological fluids, we confirmed the presence of PGE₂ in SP derived from live amebas. Why *E. histolytica* should produce a host mediator is intriguing. Given that *E. histolytica* requires AA for the biosynthesis of PGE₂ and that host diet is a rich source of AA, we speculate that the parasite is naturally selected to produce high levels of PGE₂ in the gut. In this regard, it was not unexpected that trophozoites cultured in the presence of 100 μmol/L AA for 2 hours in HBSS exhibited a robust increase in PGE₂ production. Because increased production of PGE₂ in the gut is invariably associated with pathophysiological functions, we hypothesized that high-output PGE₂ secreted by *E. histolytica* can play a significant role in the development of acute diarrhea by altering the host epithelial barrier and transport functions. Our results identify PGE₂ as the bioactive factor present in SP responsible for the disruption of the colonic epithelial TJ barrier.

We used T84 colonic monolayers grown on Transwell plates as an *in vitro* epithelial model. This cell line forms a polarized monolayer with effective TJs and with an inherent monolayer resistance similar to that observed *in vivo*.^{48–50} Moreover, T84 is one of the few transformed colonic cell lines that produce very low endogenous PGE₂,⁸ making it the cell line of choice for studying the effects of exogenous PGE₂. Because excretory and secretory components of *E. histolytica* freely interact with the colonic epithelium on the luminal side (apical side of epithelia), we investigated the effects of SP on colonic epithelial integrity by exposing the T84 monolayer to SP

on its apical side. Contrary to previous reports,⁴³ we found that secreted components consistently decreased TER. We speculate that SP collected from *E. histolytica* that were serum-starved before the collection of SP, as others have reported,⁴³ could account for the observed differences. An exogenous supply of AA is required for the parasite to produce PGE₂,¹⁵ and serum is a rich source of AA. To circumvent this problem, we cultured amebas in TYI-S-33 medium containing 20% serum and the SP was collected without subjecting amebas to serum starvation. SP was collected in HBSS after thorough washing of trophozoites, to avoid serum contamination, even though serum alone had no adverse effects on TER. Moreover, we used 0.4- μ m pore-sized polyethylene Transwell inserts as a substratum to grow T84 monolayers. In this system, T84 monolayers consistently reached a TER of $>2000 \Omega\cdot\text{cm}^2$. However, the same cells when grown on 3- μ m pore-sized polycarbonate plates, as described previously,⁴³ did not reach a TER of even $250 \Omega\cdot\text{cm}^2$ (data not shown). We propose that consistent maintenance of high TER values is critical for studies on monolayer resistance.

We confirmed the specificity of ameba-derived PGE₂ in altering TER by studying the effect of SP collected from trophozoites that were inhibited (with ASA) in their ability to synthesize PGE₂, as described previously.^{15,18} SP derived from ASA-treated amebas significantly prevented the decrease in TER. To prove specificity for PGE₂ in SP in decreasing TER, a neutralizing antibody against PGE₂ can be used. Specific neutralizing antibodies for PGE₂ are not commercially available, however, and unfortunately a neutralizing antibody procured from Cayman Chemical (catalog no. 10009814) did not yield positive results in the present study (see Supplemental Figure S4 at <http://ajp.amjpathol.org>). Nevertheless, pretreatment of colonic epithelial monolayer with EP4 receptor-specific antagonist significantly abrogated SP-induced decrease in TER, confirming the specificity for ameba-derived PGE₂ in altering TER. However, given that ASA or EP4 antagonist did not completely abrogate the decrease in TER, other ameba-secreted components may also play a role in decreasing epithelial resistance. Such molecules remain to be identified, because the major virulent components of the parasite (including CP5, amebapore, and serotonin) did not alter TER. At present, we cannot rule out a role for other putative secreted components apart from PGE₂ in altering epithelial monolayer resistance.

PGE₂ is well known for its dual role in exerting both physiological and pathophysiological functions in the gastrointestinal tract, based on amounts produced in a local milieu.⁴² To determine whether increased parasite production of PGE₂ resulted in a corresponding decrease in TER, we tested graded doses of SP on the resistance of colonic epithelial monolayer. SP followed a dose-response relationship, indicating a concentration dependency for its effects on TER. This effect was mirrored by commercially available purified PGE₂. Nonetheless, we determined the molar concentration of PGE₂ present in the high dose of SP (48 μ g) used in our experiments. Surprisingly, the concentration was approximately 4 nmol/L. However, SP followed a dose-response

in decreasing TER very similar to that of exogenous PGE₂. ASA treatment of amebas significantly decreased the amount of PGE₂ in the SP and had a corresponding effect on TER. Pretreatment of colonic epithelial monolayer with an EP4 receptor-specific antagonist significantly prevented the decrease in TER caused by a high dose of SP. Thus, we could establish the significance of ameba-derived PGE₂ signaling via the EP4 receptor of epithelial cells in decreasing TER. The potential for other known (see Supplemental Figure S1 at <http://ajp.amjpathol.org>) and unknown SP factors that can enhance the effects of PGE₂ cannot be discounted.

TER is the gold standard for the measurement of integrity of paracellular TJs. Loss of TER in contiguous monolayers strongly indicates a break in the TJ barrier for macromolecules and/or ions. In the present study, a PGE₂-induced decrease in TER corresponded with TJ permeability to ions. This effect of PGE₂ is in complete contrast to the observed macromolecule permeability when *E. histolytica* physically contacts and perturbs the epithelial monolayer.^{43,44} Thus, *E. histolytica*-induced alterations in epithelial monolayer resistance are distinct for contact-dependent versus contact-independent modes of interaction. Given that high-output PGE₂ rapidly decreases TER, we speculate that the EP4 receptors activated by PGE₂ and through which PGE₂ signals must be localized on the apical plasma membrane of colonic epithelial cells. We confirmed apical membrane localization of EP4 receptors in human colonic mucosal biopsies by immunohistochemistry and on T84 monolayers by confocal microscopy.⁵¹ We also propose that the rapidity of alteration in TER can be attributed to a sudden spatial alteration of TJ proteins, rather than to their comparatively complex transcriptional/translational regulation. Cell fractionation and immunofluorescence confocal microscopic studies confirmed spatial alteration for claudin-4. In fact, fractionation studies indicate an increased shift of claudin-4 from the membrane to the cytoskeletal fraction. Using an inhibitor for actin polymerization, we demonstrate that claudin-4 is associated with the cytoskeleton when it is dissociated from TJs. Surprisingly, claudin-4 is not colocalized with F-actin, indicating its indirect association with the actin cytoskeleton (see Supplemental Figure S5 at <http://ajp.amjpathol.org>). At present, it is not clear how the actin cytoskeleton pulls claudin-4 away from the TJ.

It is well established that the claudin family of proteins regulates TJ ion permeability. More importantly, claudin-4 regulates paracellular sodium ion permeability. Its presence at the TJ decreases sodium ion permeability across paracellular space.⁵² The present findings show selective dissociation of claudin-4 from TJ in response to ameba-derived PGE₂ or a high dose of purified PGE₂. We note that crosstalk occurs between PGE₂-induced activation of apical chloride channels (CFTR) and alteration at the TJ. More importantly, PGE₂ induced basal to apical (unidirectional) movement/flux of sodium ion across the TJ, as evident from bidirectional assessment of 20% NaCl dilution potential and by quantification of apical sodium potential. It seems that PGE₂ induces unidirectional passive flow of sodium across the TJ in response to a robust

presence of chloride on the apical side. The rapidity with which PGE₂ induces apical chloride secretion, spatial alteration of claudin-4 at TJs (occurs at 5 minutes), and the increase in apical sodium potential (occurs at 15 minutes) in the absence of activation of major membrane sodium channels is highly suggestive of this phenomenon. Thus, PGE₂ disrupts the TJ barrier for sodium ions, which, along with increased apical chloride secretion, would draw water toward the luminal side to maintain osmotic balance *in vivo*.

Taken together, these results demonstrate that the observed alteration in TJs is an effect of PGE₂ secreted by *E. histolytica*. The present findings further reinforce the concept that this parasite can initiate disease pathogenesis even in the absence of parasite-epithelial contact, and highlight a critical role for *E. histolytica*-derived PGE₂ in the onset of events that lead to diarrhea in intestinal amebiasis. Identification of EP receptor subtype 4 through which parasite-derived PGE₂ signals to alter TER widens the scope for pharmacological intervention in amebic diarrhea. This is of paramount significance, in light of the fact that amebic diarrhea often causes life-threatening complications, especially in young children. Finally, the present findings can be extrapolated to various other diarrheal diseases in which increased production of PGE₂ is a hallmark.

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