Cyclooxygenase blockade and exogenous glutamine enhance sodium absorption in infected bovine ileum

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Cole, Jeffrey, Anthony Blikslager, Elaine Hunt, Jody Gookin, and Robert Argenzio. Cyclooxygenase blockade and exogenous glutamine enhance sodium absorption in infected bovine ileum. Am J Physiol Gastrointest Liver Physiol 284: G516–G524, 2003. First published December 4, 2002; 10.1152/ajpgi.00172.2002.—We have previously shown that prostanooids inhibit electroneutral sodium absorption in Cryptosporidium parvum-infected porcine ileum, whereas glutamine stimulates electroneutral sodium absorption. We postulated that glutamine would stimulate sodium absorption via a cyclooxygenase (COX)-dependent pathway. We tested this hypothesis in C. parvum-infected calves, which are the natural hosts of cryptosporidiosis. Tissues from healthy and infected calves were studied in Ussing chambers and analyzed via immunohistochemistry and Western blots. Treatment of infected tissue with selective COX inhibitors revealed that COX-1 and -2 must be blocked to restore electroneutral sodium absorption, although the transporter involved did not appear to be the expected Na+/H+ exchanger 3 isoform. Glutamine addition also stimulated sodium absorption in calf tissue, but although this transport was electroneutral in healthy tissue, sodium absorption was electrogenic in infected tissue and was additive to sodium transport uncovered by COX inhibition. Blockade of both COX isoforms is necessary to release the prostaglandin-mediated inhibition of electroneutral sodium uptake in C. parvum-infected calf ileal tissue, whereas glutamine increases sodium uptake by an electrogenic mechanism in this same tissue. Cryptosporidium parvum; Na/H exchanger 3; cyclooxygenase-2; diarrhea

CRYPTOSPORIDIUM PARVUM is the most common enteric pathogen of young calves and is an important cause of diarrhea in animals and people worldwide (14, 31). In the United States alone, a number of large outbreaks of water-borne diarrhea has been reported in recent years; at least six of which involved >18,000 individuals (2, 24, 31). The ability of this protozoan to cause death in immunocompromised humans as well as the economic impact on the livestock industry has resulted in a National Institutes of Health panel ranking this organism as one of the three most important enteropathogens (21, 25). Unfortunately, there is no vaccine or antimicrobial agent that is presently effective; therapy involves oral or intravenous rehydration.

Cryptosporidium parvum infection has been shown to increase tissue prostaglandin concentrations both in pigs and humans, in some cases by up to 50% of baseline levels (6, 22). Prostaglandins, an integral part of the inflammatory response to infection, have been shown to inhibit sodium absorption, and their blockade with the nonspecific cyclooxygenase (COX) inhibitor indomethacin restored sodium absorption to normal (4, 4). Recent studies (13, 34, 36) have indicated the presence of two COX isoforms: one constitutive (COX-1) and one inducible (COX-2). In humans, COX-2 levels increase following infection with C. parvum, whereas COX-1 levels remain unaffected (22). Although attention has increasingly focused on the possible efficacy of inhibiting COX-2, while preserving COX-1 activity for normal homeostatic mechanisms, recent studies suggest that there may be substantial overlap in the roles of COX-1 and -2 (35).

Oral rehydration solutions are one of the main methods of maximizing fluid absorption and replenishing fluids lost with the profuse, watery diarrhea characteristic of cryptosporidiosis. Most solutions are glucose based. However, because the enterocytes that transport glucose reside on the villous tips of the small intestine and are damaged by organisms such as C. parvum, other potential substrates are being investigated. For example, glutamine has well-documented intestinotrophic effects as well as improved fluid absorption compared with glucose-based solutions in porcine cryptosporidiosis (7, 18). Previous research has also indicated that glutamine stimulates electroneutral sodium absorption in piglets (7). This vectorial transport activity is typically attributed to one of two Na+/H+ exchangers in the intestine (NHE2 and NHE3) (12). Conversely, prostaglandins inhibit NHE activity by stimulating increased intracellular cAMP levels. The present study examines the hypothesis that inhibition of a single COX isoform, most likely COX-2, would restore electroneutral sodium transport follow-

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ing infection with *C. parvum* and allow the oral administration of glutamine to be maximally effective in increasing sodium uptake.

**MATERIALS AND METHODS**

**Animals and infection.** The University Animal Care and Use Committee approved all procedures. Experimental animals were single-lineage, 1-day-old male, Holstein calves obtained from a local farm and housed in isolation facilities at the North Carolina State University College of Veterinary Medicine. Immediately after arrival, the calves were evaluated for serum colostral antibody status (Bova-S FPT Test kit, Veterinary Medical Research and Development, Pullman, WA) and fed an antibiotic-free synthetic diet (Purina Kid Milk Replacer, Purina Mills, St. Louis, MO) twice per day at a daily volume of 10% body weight. No calves were removed for failure of colostral transfer. A number of criteria was applied to the calves for exclusion from the study. No calves were removed for nongastrointestinal health problems or receiving the oral inoculum of *C. parvum* but not exhibiting diarrhea. Additionally, calves designated for the infected group, who received the oral inoculum of *C. parvum* but did not exhibit diarrhea, were to be eliminated. No animals were eliminated for this reason. Histology was examined from all calves for the presence of oocysts. Animals in the infected group with no oocysts present or, conversely, animals in the control group with oocysts present were to be eliminated. No deliberately infected calves were eliminated for this reason; several control calves were removed from the study for *C. parvum* contamination. Once mounted in the chambers, the tissues were paired for subsequent unidirectional fluxes of sodium and chloride. Initial conductance readings from the paired tissues that were >20% different from one another resulted in elimination. Several tissues, and thus treatments, were eliminated for this reason. This problem was attributed, in part, to small tears in the tissue during the stripping and mounting procedure. Some calves designated for the control group, who received inoculum vehicle only, did exhibit diarrhea. Several animals were eliminated for this reason; the cause of diarrhea was determined in all cases to be *C. parvum* contamination.

Pleasant Hill Farm (Troy, ID) provided purified *C. parvum* oocysts. Calves designated for the infected group received an oral inoculum of 10⁸ oocysts on day 7 of life; control calves received an inoculum vehicle. Previous research in this laboratory has indicated that the period of maximal diarrhea and intestinal damage is on day 4 postinfection (unpublished observations); therefore, both control and infected calves were euthanized 4 days after infection (day 11 of life) by a lethal overdose of intravenous pentobarbital sodium. After this, sections of ileum beginning 10 cm proximal to the ileocecal valve were taken for in vitro studies.

Ileal sections were opened along the antimesenteric border and bathed in an oxygenated Ringer solution. Blind dissection removed the outer muscular layers in preparation for mounting the mucosa in Ussing chambers. Additional tissue samples were formalin fixed for light microscopy and immunohistochemistry (IHC) or frozen in liquid nitrogen for Western blot analysis.

**Tissue morphology.** The formalin-fixed tissues were embedded in paraffin, cut in slices 5-μm thick, and stained with hematoxylin and eosin for analysis by light microscopy. Tissue samples from all calves were examined to determine the presence or absence of *C. parvum* organisms. Six well-oriented villi on histological sections from each animal were measured to determine mean villous height and diameter as well as crypt depth. These data were converted to measurements describing surface area, as described previously (6). Briefly, the calculations were based on the equation for the surface area of a cylinder. However, the formulas for the two ends of the cylinder were removed and replaced with the formulation for the surface area of a hemisphere, which simulates the cap of the villi. The calculation also includes a correction factor for the characteristic villous folds and variability in the frontal sections of the tissue slices.

In all animals, the infection by *C. parvum* was quantified. After hematoxylin and eosin staining, as described above, the number of oocysts per linear micrometer of villous surface was quantified. *C. parvum* forms a parasitophorous vacuole immediately subjacent to the apical membrane, with no further penetration by the organism. The quantification procedure focused on the epithelial surface and was the mean of three sections per animal. Eight animals from each group were analyzed. Control animals that exhibited *C. parvum* infection were eliminated from the study and thus were not quantified.

Inflammation due to the *C. parvum* infection was quantified by determining the extent of neutrophil infiltration into the apical segment of select well-oriented villi. With the use of a 0.01-mm² calibrated grid within the eyepiece of a light microscope superimposed over hematoxylin and eosin-stained ileal villi sections, neutrophils were quantified in three sections per animal. Eight animals from each group (control and infected) were examined.

**IHC.** For IHC, tissues were fixed in 10% neutral buffered formalin for 24 h, transferred to a 70% ethanol solution, and embedded in paraffin. Five-micrometer sections were mounted on slides, deparaffinized, and rehydrated. Slides were subsequently incubated in 3% H₂O₂, after which endogenous avidin and biotin were inhibited. After slides were further washed in PBS, they were incubated for 1 h at room temperature with a 1:50 dilution of either rabbit anti-NHE3 or -NHE2 polyclonal antibody or rabbit anti-COX-1 or -COX-2 polyclonal antibody (Chemicon; Temecula, CA). This step was not performed on negative control slides. After this, slides were incubated with goat anti-rabbit secondary antibody (Zymed; San Francisco, CA) and then labeled with aminethyl carbazole (Zymed).

**Western blot analysis.** Tissues were stored at −20°C before preparation for SDS-PAGE, at which time they were thawed at 4°C. One-gram tissue portions were added to 3 ml of chilled RIPA buffer [0.15 M NaCl, 50 mM Tris (pH 7.2), 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS], including protease inhibitors. The sample was homogenized on ice and centrifuged (2,500 g, 10 min, 4°C). The supernatant was transferred to microcentrifuge tubes and centrifuged again (10,000 g, 10 min, 4°C). Protein analysis was performed on aliquots (10 μl; Dc protein assay, Bio-Rad, Hercules, CA). Tissue extracts were mixed with an equal volume of 2× SDS-PAGE sample buffer. After 4 min of boiling, protein was separated on a 10% polyacrylamide gel, with electrophoresis carried out by standard protocols. Proteins were then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Life Science, Birminham, UK) using an electroblotting miniapparatus. Prestained molecular weight markers provided an estimate of transfer efficiency. The membrane was blocked overnight with Tris-buffered saline plus 5% dry powdered milk. The membrane was exposed for 2 h to rabbit polyclonal antibody (anti-NHE-3 or anti-COX-2; Chemicon). After the membranes were rinsed three times with Tris-buffered saline plus 0.05% Tween-20 (TBST), they were exposed to horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min.
The membranes were washed again three times with TBST and a final wash with TBS and developed for visualization of protein by the addition of enhanced chemiluminescence reagent.

**Ussing chambers.** In vitro studies in this laboratory using Ussing chamber methodology have been described in detail (5). Briefly, ileal mucosa was stripped from the muscularis and mounted in Ussing chambers with an aperture of 1.13 cm². Both tissue surfaces were bathed with 10 ml Ringer solution oxygenated with 95% O₂-5% CO₂ and maintained in water-jacketed reservoirs at 37°C. Serosal glucose (10 mM) was osmotically balanced with mucosal mannitol (10 mM). In these experiments, the tissues were stripped in either normal Ringer or Ringer containing the appropriate concentration of COX inhibitor. Additionally, the Ringer solution in the reservoirs contained the appropriate treatments before mounting the tissues.

Varying dosages and specificity of COX inhibitors were compared with a normal Ringer solution, which established the baseline. The response of various species and tissues to COX inhibition was a selective COX-1 inhibitor, SC-560 (10⁻⁶ M), or a selective COX-2 inhibitor, NS-398 (10⁻⁶ M; Cayman Chemical, Ann Arbor, MI). Tissue was incubated with the treatments for 30 min before the initial reading. After this presampling equilibration period, a 30-min period of sampling evaluated tissue response to COX blockade. After this time period, 30 mM glutamine was added to the mucosal side of the tissue, balanced with 30 mM mannitol on the serosal side, and allowed to equilibrate for 15 min. Data were then collected for an additional 30 min.

The spontaneous potential difference (PD) was measured with Ringer-agar bridges connected to matched calomel electrodes, and the PD was short circuited through Ag-AgCl electrodes, by using an automatic voltage clamp corrected for fluid resistance, for measurement of short-circuit current (Iₛₑ). After the tissue was equilibrated for 30 min, the isotopes ²²Na and ³⁶Cl were added to the mucosal or serosal reservoirs bathing paired tissues. If the conductance (G) of the tissue pairs differed by >25%, the tissues were discarded from analysis. The isotopes were then equilibrated for 20 min, at which time standards and zero time samples were taken from the reservoirs. Samples were then removed after 30 min from the side opposite that of isotope addition. After the addition of glutamine and an additional equilibration period, a second 30-min flux period was conducted.

**Isotope quantification.** Samples were counted for ²²Na in a crystal scintillation counter and for ³⁶Cl in a liquid scintillation counter. The contribution of ²²Na counts to ³⁶Cl counts was determined and compensated for. Unidirectional sodium and chloride fluxes from mucosa to serosa (Jₘₛ) and from serosa to mucosa (Jₛₘ) were calculated. From these values, a net ion flux (Jₙ) was calculated. Conductance was calculated from the PD and short-circuit current (Iₛₑ). When PD was between −1 and 1 mV, tissues were clamped at ±100 µA for 5 s and the PD was recorded to assure accurate measurement.

**Statistical analysis.** Densitometry and villi morphology data were analyzed using an unpaired t-test. Electrical and electrolyte flux data were analyzed by a two-way ANOVA for multiple comparisons using group (control vs. infected) and treatment as variables, followed by a post hoc Tukey’s test to determine differences among treatments (Sigma Stat, Jandel Scientific, San Rafael, CA). Significance was declared at P < 0.05.

### RESULTS

**Histology and molecular biology.** Villous atrophy and crypt hyperplasia are two well-substantiated characteristics of intestinal tissue during cryptosporidial infection (15, 23, 26). Ileal mucosa from infected calves examined at the peak of diarrhea demonstrated shortened villi and elongated crypts (Fig. 1; P < 0.05). However, surface area was not affected due to the significant increase (P < 0.05) in the width of villi following infection (data not shown).

![Fig. 1. Villous morphology in control and infected tissue collected 4 days after infection. Infection caused a significant reduction (P < 0.05) in the villous height, attributable to villous atrophy characteristic of Cryptosporidium parvum-induced damage. Crypt depth significantly increased (P < 0.05) following infection, indicative of crypt hyperplasia in response to injury.](http://ajpgi.physiology.org/Downloadedfrom)
Calves infected with *C. parvum* had 12.7 ± 2.2 oocysts/100 μm of epithelium. Control calves were not measured, because any calves assigned to the control group that displayed *C. parvum* oocysts were eliminated from the study.

Inflammation, as determined by histological neutrophil counts, was significantly higher in the infected tissue compared with the control tissue (*P* < 0.05). Animals infected with *C. parvum* had 367 ± 54 neutrophils/mm² within the apical portion of villi, whereas control animals had 71 ± 33 neutrophils/mm².

Previous research has indicated that in the intestinal tissue of most species, COX-1 is constitutively expressed in healthy tissue, perhaps acting in a "housekeeping" role, whereas COX-2 is induced in response to inflammatory stimuli (13, 17, 34, 36). However, IHC conducted in the present study revealed the constitutive presence of both isoforms. In healthy tissue, the enzymes were located along the length of the villi, with the heaviest concentration in the villous tip (Fig. 2, A and C). Interestingly, the COX-1 isoform was also concentrated in the goblet cells, whereas COX-2 was absent from this cell population. In the infected animals, the characteristic villous blunting was noted with COX-1 and -2 concentrated in the tips of villi, particularly COX-2 (Fig. 2, B and D). Neither COX isoform was observed in the crypt enterocytes in either the healthy or infected animals. Western blot analysis

![Image](http://ajpgi.physiology.org/download.png)

**Fig. 2.** Tissue was sampled from both control and infected calves and analyzed immunohistochemically for the presence of cyclooxygenase (COX) isoforms. 

- **A** (COX-1) and **C** (COX-2): tissue collected from control animals, with both isoforms populating the epithelial cells the length of the villus, with less enzyme expressed in the crypt cells. 
- **B** (COX-1) and **D** (COX-2): tissue collected from infected animals. Both isoforms are present at higher concentrations in the villous tips, with none found in the crypts. Control tissue was imaged at a ×10 magnification; Infected tissue was imaged at ×20 magnification.
confirmed the presence of COX-1 and -2 in both control and infected tissue (Figs. 3 and 4).

Intestinal absorption of NaCl is normally driven by a neutral NHE. Of the NHE family of transporters, only NHE2 and NHE3 have been associated with vectorial transport. Immunohistochemistry revealed the presence of NHE3 in healthy ileal tissue, and it was limited to the terminal portion of the villi (Fig. 5A). The location of NHE3 would appear to indicate that the exchanger is expressed only in mature enterocytes, because the protein was localized to the tips of the villi. Although NHE3 was identified in the apical membrane, most was found inside the cell. As Kurashima et al. (20) reported, ~90% of NHE3 protein will be found in juxtanuclear accumulation complexes. Infected tissue did not appear to contain NHE3 (Fig. 5B). This lack of NHE3 in infected tissue was supported by Western blots (Fig. 6). Tissues were also examined for the presence of NHE2 proteins. Neither control nor infected animals demonstrated the presence of NHE2 (data not shown).

Sodium and chloride transport studies. In both piglets and calves, infection with C. parvum reduces villus absorption of sodium and chloride (7, 9). In this study, infection completely abolished net sodium absorption (Fig. 7), whereas net chloride absorption was not significantly different from control. The infection also increased the $I_{sc}$ (Fig. 7; $P < 0.05$) and decreased the tissue conductance (Fig. 7; $P < 0.05$). Administration of indomethacin, NS-398, or SC-560 restored sodium absorption in infected tissue to levels no different from untreated control tissues (Fig. 7; $P > 0.05$). These increases in net sodium absorption were not associated with significant changes in $I_{sc}$ (Fig. 7), indicating the COX inhibitor-induced increase in sodium absorption was electroneutral. Administration of COX inhibitors did not alter net sodium or chloride fluxes or $I_{sc}$ in control tissue.

Preliminary data (not shown) indicated that administration of SC-560 or NS-398 at $10^{-6}$M did not improve sodium absorption in infected animals compared with normal Ringer solution alone. However, the non-selective COX inhibitor indomethacin ($10^{-6}$M) resulted in a significant increase ($P < 0.05$) in sodium flux compared with infected tissue in normal Ringer solution alone (Fig. 7). However, simultaneous addition of both selective inhibitors at $10^{-6}$M was equally as effective in restoring sodium absorption as indo-

Fig. 3. Tissue was sampled from both control and infected calves and analyzed by Western blot for the presence of COX-1. A: control lane is representative of $n = 3$; infected lane is representative of $n = 2$. B: intensity of immunoreactive bands was quantified using scanning densitometry software (Scanalytics). Values are density means ± SE; control, $n = 3$; infected, $n = 2$; means with differing letters are significant at $P < 0.05$.

Fig. 4. Tissue was sampled from both control and infected calves and analyzed by Western blot for the presence of COX-2. A: each lane (control and infected) is representative of $n = 3$ for each treatment group. The appearance of similar levels of COX-2 in control and infected tissues is suggestive of constitutive expression of this protein in calf ileum. B: intensity of immunoreactive bands was quantified using scanning densitometry software (Scanalytics). Values are density means ± SE ($n = 3$); means with differing letters are significant at $P < 0.05$. 

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methacin at $10^{-6}$ M, suggesting that inhibition of both COX-1 and -2 is required to restore sodium absorption following *C. parvum* infection. Higher doses of the selective COX inhibitors ($10^{-5}$ M) also restored sodium absorption when added individually or together (Fig. 7), suggesting a lack of specificity at the higher doses. Islam et al. (18) demonstrated that glutamine increases electrolyte absorption; but the mechanism by which it does so appears to vary from species to species. In piglets, glutamine appears to stimulate an electroneutral sodium transport mechanism, whereas in calves, electrogenic transport was noted (7, 9). In the healthy tissue examined in the present study, glutamine administration (30 mM) increased sodium absorption compared with normal Ringer solution alone ($P < 0.05$; compare Figs. 7 and 8). The stimulatory effect of glutamine was limited to increased sodium absorption, with no concurrent alteration of net chloride absorption or $I_{sc}$ ($P < 0.05$; Fig. 8), indicating an electroneutral mechanism of action. Treatment of the healthy tissue with COX inhibitors did not increase sodium absorption compared with glutamine alone and even decreased sodium absorption in the presence of SC-560 ($10^{-5}$ M) or SC-560 and NS-398 ($10^{-6}$ M; Fig. 8).

In infected tissue, glutamine administration (30 mM) increased sodium absorption compared with normal Ringer solution alone ($P < 0.05$; compare Figs. 7 and 8). In contrast to the control tissue, the addition of COX inhibitors further increased sodium absorption compared with glutamine alone (Fig. 8). In further contrast to control tissue and regardless of inhibitor treatment, the addition of glutamine to infected tissue induced an increase in $I_{sc}$ ($P < 0.05$; Fig. 8) equal to the increase in net sodium transport, indicating electrogenic sodium transport activity. This was confirmed by the lack of response in chloride movement.

**DISCUSSION**

The most significant findings of this study were that, despite the villous atrophy and absence of the conventional electroneutral transporters (NHE2 and NHE3), neutral sodium absorption was fully expressed in infected ileum after treatment with COX inhibitors. Fur-
thermore, glutamine stimulated sodium absorption equally in control and infected ileum, but by different mechanisms. Thus these results suggest an adaptive response in infected tissue, with an upregulation of novel sodium transport mechanisms.

The significant increase in electrically neutral sodium uptake in infected tissue following inhibition of prostaglandins by COX blockers occurred despite the absence of IHC or Western blot evidence of NHE2 and NHE3, the neutral NHEs typically found on the apical membrane in ileal tissue (10, 16). After C. parvum infection, the intestine displays severe villous atrophy as a result of both pathogen damage and as a result of villous contraction, a protective mechanism, to reduce the surface area of damaged mucosa. After clearance of the organism and recovery, the intestine restores the villi to the normal height and repopulates them with immature enterocytes whose initial primary role is the restoration of a physical barrier. Thus cells on the villi may not have acquired transporters such as NHE3 associated with mature epithelium. Therefore, an alternate sodium transporter in either immature villous or crypt epithelium may be involved; recently, Rajendran et al. (28) and Binder et al. (8) described a novel

Fig. 7. Sodium and chloride flux and electrical data were measured in both control and infected tissues that were collected 4 days after infection and treated with COX inhibitors. Inhibition of COX activity restored sodium absorption in infected tissue to levels seen in healthy tissue. Inhibition of COX activity did not alter the \( J_{\text{sc}} \) response, although conductance \( (G) \) did decrease compared with the healthy tissue. Values are means ± SE. NS-398, selective COX-2 inhibitor; SC-560, selective COX-1 inhibitor. Means within a column with differing superscripts differ \((P < 0.05)\). Indomethacin was added to achieve a concentration of \( 10^{-6} \) M; NS-398 and SC-560 were added to achieve final concentrations of \( 10^{-5} \) M. Both NS-398 and SC-560 were added together to achieve concentrations of \( 10^{-5} \) and \( 10^{-6} \) M. \( J_{\text{sc}} \), net flux.

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\text{Group} & \text{n} & \text{\( J_{\text{net}} \)} (\mu\text{Eq/cm}^2\text{h}) & \text{\( J_{\text{net}} \)} (\mu\text{Eq/cm}^2\text{h}) & \text{\( J_{\text{net}} \)} (\mu\text{Eq/cm}^2\text{h}) & \text{\( J_{\text{net}} \)} (\mu\text{Eq/cm}^2\text{h}) \\
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\text{Control - Ringer} & 8 & 2.4 ± 0.5^a & 1.5 ± 0.2^b & -0.2 ± 0.1^a & 27.1 ± 1.1^a \\
+ Indomethacin & 7 & 1.3 ± 0.6^a & 1.8 ± 0.2^b & -0.4 ± 0.1^a & 30.1 ± 1.2^a \\
+ NS-398 & 8 & 2.0 ± 0.5^a & 1.2 ± 0.4^bc & -0.2 ± 0.2^a & 29.8 ± 1.4^a \\
+ SC-560 & 7 & 1.2 ± 0.8^a & 1.1 ± 0.3^c & -0.2 ± 0.1^c & 33.4 ± 1.5^a \\
+ NS-398 + SC-560 (10^{-5} M) & 7 & 2.0 ± 0.7^a & 1.8 ± 0.5^bc & -0.4 ± 0.1^a & 32.8 ± 2.0^a \\
+ NS-398 + SC-560 (10^{-6} M) & 7 & 1.2 ± 0.6^a & 0.6 ± 0.8^c & -0.3 ± 0.1^c & 31.6 ± 2.2^a \\
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\text{Infected -Ringer} & 5 & 0.0 ± 0.4^a & 0.5 ± 0.5^c & 0.4 ± 0.1^b & 14.1 ± 0.4^c \\
+ Indomethacin & 7 & 2.5 ± 0.5^a & 1.7 ± 0.3^a & 0.2 ± 0.1^b & 14.3 ± 1.0^b \\
+ NS-398 & 4 & 2.6 ± 0.3^a & 2.0 ± 0.4^ab & 0.1 ± 0.2^b & 15.8 ± 0.5^bc \\
+ SC-560 & 4 & 2.6 ± 0.5^a & 1.6 ± 0.4^ab & 0.2 ± 0.2^b & 17.7 ± 0.7^bc \\
+ NS-398 + SC-560 (10^{-5} M) & 4 & 2.2 ± 0.9^a & 2.2 ± 0.8^ab & 0.1 ± 0.1^b & 20.7 ± 2.4^c \\
+ NS-398 + SC-560 (10^{-6} M) & 5 & 2.8 ± 0.8^a & 1.9 ± 0.6^ab & 0.0 ± 0.1^b & 18.6 ± 2.3^c \\
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Fig. 8. Sodium and chloride flux and electrical data were measured in both control and infected tissue that was collected 4 days after infection and treated with COX inhibitors and glutamine. Glutamine significantly increased sodium absorption in both healthy and infected tissue \((P < 0.05)\). Glutamine administration significantly \((P < 0.05)\) increased \( J_{\text{sc}} \) in infected tissue compared with the healthy tissue, although this response was independent of COX inhibition. Conductance was not altered by glutamine treatments. For each panel, values are means ± SE, and means within a column with differing superscripts differ \((P < 0.05)\). NR, normal Ringer solution; IR, Ringer solution containing indomethacin; NS-398, selective COX-2 inhibitor; SC-560, selective COX-1 inhibitor. Glutamine was added to achieve a concentration of 30 mM. Indomethacin was added to achieve a concentration of \( 10^{-6} \) M; NS-398 and SC-560 were added to achieve final concentrations of \( 10^{-5} \) M. Both NS-398 and SC-560 were added together to achieve concentrations of \( 10^{-5} \) and \( 10^{-6} \) M. \( J_{\text{sc}} \), net flux.

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\text{Group} & \text{n} & \text{\( J_{\text{net}} \)} (\mu\text{Eq/cm}^2\text{h}) & \text{\( J_{\text{net}} \)} (\mu\text{Eq/cm}^2\text{h}) & \text{\( J_{\text{net}} \)} (\mu\text{Eq/cm}^2\text{h}) & \text{\( J_{\text{net}} \)} (\mu\text{Eq/cm}^2\text{h}) \\
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\text{Control + glutamine} & 8 & 4.5 ± 1.0^a & 2.1 ± 0.5^a & -0.1 ± 0.2^a & 28.4 ± 1.3^a \\
+ Indomethacin + glutamine & 7 & 3.3 ± 0.8^b & 3.3 ± 0.6^a & -0.4 ± 0.2^a & 31.2 ± 0.8^ab \\
+ NS-398 + glutamine & 8 & 3.6 ± 0.7^bc & 2.3 ± 0.5^a & -0.4 ± 0.3^a & 30.6 ± 1.2^ab \\
+ SC-560 + glutamine & 8 & 1.5 ± 0.5^b & 1.8 ± 0.5^a & -0.3 ± 0.2^a & 34.4 ± 1.7^a \\
+ NS-398 + SC-560 (10^{-5} M) & 7 & 4.5 ± 1.0^a & 4.2 ± 0.9^a & -0.6 ± 0.2^a & 32.1 ± 1.5^a \\
+ glutamine & 8 & 2.9 ± 1.0^c & 1.0 ± 1.0^cd & -0.1 ± 0.2^a & 33.8 ± 2.6^b \\
+ NS-398 + SC-560 (10^{-6} M) & 7 & 4.5 ± 0.9^a & 1.7 ± 0.7^ac & 1.2 ± 0.2^b & 17.2 ± 0.8^c \\
+ glutamine & 5 & 4.5 ± 0.9^a & 1.7 ± 0.7^ac & 1.2 ± 0.2^b & 17.2 ± 0.8^c \\
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NHE in crypt epithelium, which has been associated with sodium and H2O absorption. A more likely alternative may be an electroneutral sodium-bicarbonate cotransporter. Both electrogenic and electroneutral isoforms (NBC-1 and NBCn1) have recently been reported in murine duodenal tissue (27, 30). Further study will be necessary to determine whether any of these mechanisms are present in calf ileum and upregulated in the infection.

Previous studies of both pig and calf models of cryptosporidiosis have shown that elevated tissue levels of prostaglandins inhibit electroneutral sodium absorption in this infection (5, 9). Because of the potential deleterious effects on the mucosal barrier caused by inhibition of COX-1 and the housekeeping prostaglandins, one of the primary objectives of this study was to determine whether selective inhibition of one of the COX isoforms was sufficient to restore neutral sodium absorption. However, the lack of response seen when either COX-1 or -2 were inhibited alone at an inhibitor concentration of 10^-6 M indicates that this is probably not sufficient for effectively treating diarrhea. Instead, inhibition of both COX isoforms using SC-560 and NS-398 together at 10^-6 M was required to restore sodium absorption. The results of this study, as well as preliminary work conducted in this laboratory, indicate a loss of specificity of the COX inhibitors NS-398 and SC-560 as the concentration increases above 10^-6 M. Addition at 10^-5 M of either inhibitor resulted in flux data identical to that obtained when the nonspecific COX inhibitor indomethacin was added, suggesting a loss of specificity at this higher dose. Thus this novel neutral sodium absorptive mechanism appears to be controlled by both COX-1- and -2-elaborated prostaglandins.

In healthy tissue, glutamine administration induces sodium uptake, a response that has been well documented (1). Glutamine added to control calf tissue induced a significant increase in net sodium absorption. The absence of an I_sc response indicates the stimulation of an electroneutral transporter, which could be NHE3, because IHC indicates its presence on the tips of villi. This result differs from a previous study in control calf ileum in which a lower dose of glutamine (10 mM) induced an I_sc response equivalent to the net increase in sodium absorption (9). One possible explanation for this apparent discrepancy may lie in the higher dose of glutamine presently used. For example, Rhoads et al. (29) showed a dose dependency of glutamine-stimulated electroneutral sodium absorption in piglet intestine, which became apparent only with glutamine concentrations greater than 20 mM. The higher glutamine concentration likely results in greater cellular uptake and metabolism to CO2, which then stimulates sodium/hydrogen exchange activity (7, 11, 28).

Furthermore, glucose stimulation of sodium-dependent transport directly triggers NHE3 activity by altering intracellular pH (32). Thus it could be speculated that the increased sodium uptake via sodium/hydrogen exchange could sufficiently alter the sodium gradient across the mucosal membranes to reduce or eliminate sodium-substrate cotransport activity. Indeed, previous studies with cholera toxin, which inhibits NHE3, showed a heightened sodium-substrate transport response attributed to an altered sodium gradient.

A decrease in conductance was noted when adding glutamine to infected tissue, despite the fact that Na absorption would typically increase conductance as a result of opening of apical membrane Na channels. However, the effect of opening Na channels may be outweighed by effects of the infection and villous repair cycle on paracellular pathways. In particular, damaged tissue undergoes villous contraction and epithelial restitution with concurrent tight apposition of paracellular spaces. Thus, despite opening of apical membrane Na channels, the effect of repair on reducing paracellular conductance likely overrides the effects of transport on transcellular pathways, resulting in a net decrease in conductance.

Because 30 mM glutamine also stimulated neutral Na/H exchange, which was inhibited by prostaglandins in the infected piglet model (7), we reasoned that glutamine would be most effective in stimulating neutral sodium absorption when combined with prostaglandin inhibition. However, although there was a response to glutamine and indomethacin in infected tissue (Fig. 7), the mechanism by which glutamine stimulated sodium absorption was electrogenic. Thus 30 mM glutamine stimulates sodium absorption by distinctly different mechanisms in healthy versus infected tissue in calves. The fact that this electrogenic glutamine transport is prostaglandin independent may be an important adaptive response, because the transporter will still be operational during the inflammatory response following infection, when tissue prostaglandin levels are increased. Blikslager et al. (9) demonstrated the upregulation of the amino acid transport system ASC in the crypts of Cryptosporidium-infected bovine ileum. The glutamine stimulation of the electrogenic transporter in the present study resulted in similar increases in sodium J_net and I_sc to those reported by Blikslager et al. (9).

On the basis of the results described above, it is apparent that simply inhibiting one of the two COX isoforms is insufficient to treat cryptosporidiosis in calves. However, blockade of both COX enzymes resulted in the restoration of an electroneutral sodium transporter, which would be expected to increase fluid absorption, an important part of any treatment for this and many other diarrheal diseases. The studies discussed here suggest that this electroneutral transporter is not an NHE, as might be assumed. Instead, it is more likely another sodium-dependent, electrically silent absorptive mechanism such as a sodium-bicarbonate cotransporter. This response, coupled with the glutamine-induced stimulation of electrogenic sodium transport activity, has great potential as a treatment for not only C. parvum-induced diarrhea but possibly other diarrheal diseases characterized by villous atrophy and inflammation.
REFERENCES