Nitric oxide synthase stimulates prostaglandin synthesis and barrier function in *C. parvum*-infected porcine ileum


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Gookin, Jody L., Laurel L. Duckett, Martha U. Armstrong, Stephen H. Stauffer, Colleen P. Finnegan, Michael P. Murtaugh, and Robert A. Argenzio. Nitric oxide synthase stimulates prostaglandin synthesis and barrier function in *C. parvum*-infected porcine ileum. *Am J Physiol Gastrointest Liver Physiol* 287: G571–G581, 2004. First published May 20, 2004; 10.1152/ajpgi.00413.2003.—Cell culture models implicate increased nitric oxide (NO) synthesis as a cause of mucosal hyperpermeability in intestinal epithelial infection. NO may also mediate a multitude of subepithelial events, including activation of cyclooxygenases. We examined whether NO promotes barrier function via prostaglandin synthesis using *Cryptosporidium parvum*-infected ileal epithelium in residence with an intact submucosa. Expression of NO synthase (NOS) isoforms was examined by real-time RT-PCR of ileal mucosa from control and *C. parvum*-infected piglets. The isoforms mediating and mechanism of NO action on barrier function were assessed by measuring transepithelial resistance (TER) and eicosanoid synthesis by ileal mucosa mounted in Ussing chambers in the presence of selective and nonselective NOS inhibitors and after rescue with exogenous prostaglandins. *C. parvum* infection results in induction of mucosal inducible NOS (iNOS), increased synthesis of NO and PGE₂, and increased mucosal permeability. Nonselective inhibition of NOS (N⁴,N⁴-dinitro-l-arginine methyl ester) inhibited prostaglandin synthesis, resulting in further increases in paracellular permeability. Baseline permeability was restored in the absence of NO by exogenous PGE₂. Selective inhibition of iNOS [L-NAME-(1-iminoethyl)-l-lysine] accounted for ~50% of NOS-dependent PGE₂ synthesis and TER. Using an entire intestinal mucosa, we have demonstrated for the first time that NO serves as a proximal mediator of PGE₂ synthesis and barrier function in *C. parvum* infection. Expression of iNOS by infected mucosa was without detriment to overall barrier function and may serve to promote clearance of infected enterocytes.

*Cryptosporidium parvum*; permeability

The single layer of epithelium lining the small intestine provides a defensive barrier that restricts ingress of luminal aggressive factors while selectively absorbing the majority of nutrients, electrolytes, and water that sustain life. These barrier and absorptive functions are particularly vulnerable to microbial pathogens with tropism for intestinal epithelium, such as rotavirus, enteropathogenic *Escherichia coli*, and *Cryptosporidium parvum*. Although these infections hasten the loss of villous enterocytes, epithelial continuity is frequently preserved by hyperplastic crypts that provide a continuous source of replacement enterocytes to the villi. In contrast, a persistent source of barrier dysfunction in intestinal infection appears to arise from selective increases in epithelial paracellular permeability. Transmucosal hyperpermeability has been consistently demonstrated in infants and human immunodeficiency virus patients with diarrhea caused by these agents (12, 14, 48), in animal models of intestinal infection (4, 19), and after infection of epithelial monolayers in culture (1, 13, 18, 38). Increases in paracellular permeability may contribute to fluid losses and facilitate translocation of luminal factors in the subepithelium that perpetuate mucosal inflammation. Although much has been learned regarding mediators and mechanisms of disruption of paracellular permeability, less is understood regarding the local mechanisms responsible for sustaining barrier function during intestinal infection.

Nitric oxide (NO) is consistently elevated in patients with infectious diarrhea (27) and mediates demonstrable but paradoxical effects on paracellular permeability in a variety of experimental models. Cell culture models suggest that NO would mediate barrier disruption in intestinal epithelial infection because both normal and infected intestinal epithelia subjected to induction of NO synthesis or exogenous NO undergo increases in paracellular permeability (7, 34, 40, 42, 44). In contrast, in vivo studies performed with normal intestine (22, 23, 24) or during the acute phase of intestinal injury (25, 37) support the view that NO preserves barrier function by mechanisms related to subepithelial events, such as maintenance of mucosal blood flow (36), inhibition of leukocyte adhesion (26), and modulation of mast cell reactivity (22). However, in the presence of mucosal inflammation, the role of NO-mediated effects is less clear, with NO promoting barrier function in some studies (33) and mediating barrier disruption in others (45). Studies have yet to define the local effect of NO on paracellular permeability of an entire intestinal mucosa in the presence of both epithelial infection and the resulting subepithelial inflammatory infiltrate.

In the presence of inflammation, NO can combine with superoxide to generate potent free radicals, such as peroxynitrite, that exacerbate epithelial injury (35, 39, 43, 47). On the other hand, reactive nitrogen species have also been demonstrated to activate cyclooxygenase (COX) in enzymatic studies and cell culture models (15). We have previously shown that synthesis of prostaglandins (PG) by ileal mucosa from *C. parvum*-infected piglets is increased and mediates diarrhea via inhibition of NaCl absorption and stimulation of anion (Cl⁻ or

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Table 1. Specific primer sequences and sources used in real-time RT-PCR and their resulting amplification product sizes and melting temperatures

<table>
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<th>GenBank No.</th>
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<th>Reverse (5’ to 3’)</th>
<th>Product Size, bp</th>
<th>Tm</th>
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<td>Cyclophilin</td>
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<tr>
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<td>eNOS</td>
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<td>gccgaagacgctattgacg</td>
<td>150</td>
<td>85.3–85.5</td>
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<tr>
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<td>81</td>
</tr>
<tr>
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<td>iNOS</td>
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<td>ggcacagggcctgctagg</td>
<td>99</td>
<td>85.2–85.5</td>
</tr>
</tbody>
</table>

eNOS, nNOS, and iNOS, endothelial, neuronal, and inducible nitric oxide synthase, respectively; Tm, melting temperature.
infected piglets, mucosal sheets were incubated for 300 min in the Ussing chamber in the presence or absence of 10 mM Nω-nitro-l-arginine methyl ester (l-NAME) on the serosal side. Tissues were removed from the Ussing chamber and placed in Trump’s 4F:1G fixative at 4°C. Samples were processed for transmission electron microscopy using standard techniques.

NO and eicosanoid analyses. Total NO2 + NO3 concentration was measured in urine and serum samples taken from infected and control piglets after conversion of NO3 to NO2 by nitrate reductase with detection of NO2 using a commercial kit (Griess Assay; Cayman Chemical, Ann Arbor, MI). For eicosanoid analyses, paired samples were taken from the serosal chamber solution, gassed with N2, and frozen in liquid N2. Samples were stored at −20°C before assay. Samples were analyzed for concentrations of PGE2, 6-keto-PGF1α, (the stable metabolite of PGL2), and thromboxane (TX) B2 (the stable metabolite of TXA2) using commercial ELISA kits according to the manufacturer’s instructions (Biomedical Technologies, Stoughton, MA). Because of baseline differences in eicosanoid synthesis by control and infected mucosa and the variable influence of stripping and mounting of mucosa on initial eicosanoid synthesis, baseline samples were collected 30 min after tissue mounting for eicosanoid assay. Baseline eicosanoid concentrations were subtracted from concentrations measured after 300 min of exposure to each treatment.

Assessment of NO2 effects on barrier function. All treatments were added to the Ussing chamber 15 min after mounting of the mucosa (acclimation period) and allowed to remain in contact with the mucosa for 300 min. The following treatments were evaluated in this study: the nonselective NOS inhibitors l-NNAME (10 mM serosal) and l-NAME-monomethyl arginine (l-NMMA; 1 mM serosal); the selective iNOS inhibitors l-Nω-(1-iminomethyl)-l-lysine (l-NIL; 30 and 100 μM serosal) and aminoguanidine (5 mM serosal); and the reportedly selective nonselective NOS inhibitor l-NMMA [26]. Deoxycholate was then replaced by Ringer solution. Brieﬂy, the villous epithelium was removed by transient exposure of the chambered mucosa to deoxycholate (1.5 mM for 15 min; Sigma, St. Louis, MO). Deoxycholate was then replaced by Ringer solution. Paired tissues from each piglet were allowed to restitute for 3 h in the presence and absence of l-NNAME (10 mM serosal). Tissues were removed from the Ussing chamber, ﬁxed in formalin, parafﬁn embedded, sectioned at 5 μm, and stained with H&E for examination by light microscopy. With the use of an ocular micrometer, the linear length of villus perimeter and linear length of denuded villus were measured for 5-well-oriented villi and used to calculate the percent villus reepithelialization of each tissue. All measurements were performed without knowledge of treatment group.

Data analysis. Data are reported as means ± SE. For all analyses, P ≤ 0.05 was considered signiﬁcant. One-way ANOVA and a post hoc Tukey’s test, two-way repeated-measures ANOVA, or Student’s paired t-test was used to compare differences between treatment and control tissues (SigmaStat; Jandel Scientiﬁc, San Rafael, CA). Number of pigs receiving treatment = n.

RESULTS

Infection of neonatal piglets with C. parvum results in villus atrophy and diarrhea. Piglets were killed on days 3–5 postinfection. This time period corresponds to peak epithelial infection with C. parvum and liquid diarrhea (4, 5). Ileal mucosa from time-matched control piglets was comprised of elongated villi lined by vacuolated epithelium. In C. parvum infection, sporozoites parasitized enterocytes along the villus tips, resulting in enhanced epithelial cell loss and marked villus atrophy (villus height = 638 ± 39 μm control, 121 ± 7 μm infected; P < 0.001 1-way ANOVA).

NO synthesis is increased in piglets with C. parvum infection. To determine whether endogenous NO synthesis is increased in piglets with C. parvum infection, the oxidative metabolites of NO (NO2 and NO3) were measured in serum and urine samples taken from control and infected animals on days 3–5 postinfection (Fig. 1). The concentration of NO metabolites in serum was not signiﬁcantly different between control and infected animals. However, greater synthesis of NO by infected animals was demonstrated by a signiﬁcantly larger concentration of NO metabolites excreted in urine.

NO contributes to barrier function in intestinal C. parvum infection. To assess the effect of NO on intestinal barrier function in the infection, sections of ileal mucosa from control and infected piglets were incubated in Ussing chambers. In infected mucosa, baseline TER was signiﬁcantly lower and the passive serosal-to-mucosal ﬂux of 22Na+ was signiﬁcantly higher than for control mucosa (Figs. 2 and 3). In the absence of treatment, both infected and control mucosa maintained stable TER values for over 315 min of incubation. After addition of the nonselective NOS inhibitor l-NNAME to the serosal bath of control tissue, TER and serosal-to-mucosal ﬂux of 22Na+ were unaffected. In contrast, TER of infected tissue progressively deteriorated, and there was a signiﬁcant increase in the serosal-to-mucosal ﬂux of 22Na+ (Figs. 2 and 3).

Mucosal-to-serosal ﬂux of [3H]mannitol was likewise increased by the addition of l-NNAME to infected tissue (μmol·cm−2·h−1 = 0.28 ± 0.04 no treatment (no Tx); 0.31 ± 0.03 l-NNAME, n = 8 each; P < 0.01, Student’s paired t-test), and signiﬁcant inhibition of TER was also obtained with the nonselective NOS inhibitor l-NMMA (μΩ·cm−2·mucosa after 300 min = 25 ± 1.1 no Tx; 19 ± 0.9 l-NMMA (1 mM), n = 8 each; P < 0.01, Student’s paired t-test). Incubation of control or infected mucosa with l-NNAME, an inactive isomer of l-NNAME, was without effect on TER (Fig. 2, inset).

Hypertrophy and hyperplasia were observed in villus tips of infected piglets (Fig. 2, inset).
NO promotes paracellular integrity in C. parvum infection. Decline in barrier function of infected mucosa treated with L-NAME could result from either arrest of epithelial replacement (restitution) or loss of paracellular integrity. To determine which mechanism was responsible, infected and control tissues were incubated in the presence or absence of L-NAME and removed from the chamber after 300 min for examination by light microscopy. In the absence of treatment, control and infected epithelia were indistinguishable in appearance from their freshly obtained counterparts and were lined by a continuous, well-apposed layer of enterocytes (Fig. 4). In contrast, after incubation with L-NAME, infected mucosa showed dilation of intercellular space and expansion of the lamina propria. Only a minor amount of intercellular space dilation and expansion of lamina propria was visible in L-NAME-treated, uninfected mucosa. To more closely examine the nature of the barrier defect resulting from blockade of NO, transmission electron microscopy was performed on infected epithelium incubated in the presence and absence of L-NAME. In untreated epithelium, there was close apposition of the lateral membranes, whereas L-NAME-treated epithelium showed marked dilation of the lateral intercellular space below the tight junction (Fig. 5).

Infected mucosa treated with L-NAME remained covered by a confluent layer of epithelium, suggesting that ongoing restitution was not interrupted by blockade of NOS. To ensure that arrest of restitution would be demonstrable over the time period of our studies, restitution by infected mucosa was blocked by incubation with cytochalasin D (3 × 10−5 M), an inhibitor of actin polymerization. Such treatment resulted in demonstrable increases in exposure of villous basement membrane over the 300-min duration of incubation. We additionally quantified the underlying restitution rate of the infected epithelium in the presence and absence of L-NAME by measuring the degree of villous reepithelialization achieved after denudation by a low concentration of deoxycholate (1.5 mM for 15 min). The rate of restitution of infected mucosa was not impaired by the presence of L-NAME [%reepithelialization = 76 ± 9 no Tx; 86 ± 7 L-NAME (10 mM); n = 6 each; Fig. 6].

NO promotes barrier function via PG synthesis in C. parvum infection. Akin to our observations on the effects of L-NAME, we have previously demonstrated that TER of C. parvum-infected and not control mucosa is significantly inhibited by incubation with the nonselective COX inhibitor Indomethacin (3). Furthermore, the barrier-maintaining properties of PG are attributed to paracellular effects related to stimulation of Cl− secretion that promote withdrawal of Na+ and water from the paracellular spaces of the crypt and inhibition of neutral NaCl absorption, resulting in decreased paracellular water absorption by the villus (3, 8, 16). We therefore hypothesized that NO promotes barrier function in C. parvum infection by mediating the synthesis of endogenous PG. Thus we measured PGE2, 6-keto-PGF1α (the stable metabolite of PGI2), and TXB2 (the stable metabolite of TXA2) production by control and infected mucosa after incubation in Ussing chambers in the presence of L-NAME, Indomethacin (Indo), or both (Table 2). Production of PGE2 was significantly greater in infected mucosa. In the presence of L-NAME, synthesis of all three eicosanoids by both control and infected mucosa was inhibited significantly. To determine whether PG depletion was mediating the effect of L-NAME on barrier function in the infection, the ability of mucosa to

**Fig. 2.** Transepithelial electrical resistance (TER) of control and C. parvum-infected ileal mucosa (days 3–5 postinfection) mounted in Ussing chambers. After a 15-min acclimation period, treated tissues were exposed serosally to the nonselective NO synthase (NOS) inhibitor Nω-nitro-arginine methyl ester (L-NAME; 10 mM) for 300 min. In infected mucosa, L-NAME resulted in significant reduction in TER (***P < 0.001, 2-way repeated-measures ANOVA). The inactive D-isomer of NAME had no effect on TER (ns). **Fig. 3.** Passive flux of 22Na+ from serosa to mucosa (Jmuc) of control and C. parvum-infected ileal mucosa mounted in Ussing chambers. Fluxes were performed after 4 h of incubation in the absence (No Tx) or presence of the nonselective NO synthase (NOS) inhibitor L-NAME (10 mM applied to the serosal reservoir). Flux of 22Na+ was significantly greater in infected mucosa compared with control (**P < 0.001, Student’s t-test). Flux of 22Na+ was significantly increased in infected mucosa after treatment with L-NAME (***P < 0.05, Student’s t-test); n = no. of piglets. NS, not significant.
maintain TER was determined during incubation with Indo alone ($5 \times 10^{-6}$ M) or in combination with l-NAME (10 mM). Indo inhibited TER and dilated paracellular pathways of infected mucosa in a manner analogous to that of l-NAME. Neither inhibitor achieved a significant reduction in TER of control mucosa ($47 \pm 3.6$ (n = 12), control + l-NAME = $40 \pm 2.3$ (n = 12), control + Indo = $47 \pm 3$ (n = 12), control + l-NAME + Indo = $41 \pm 3$ (n = 1), infected + Indo = $41 \pm 3$ (n = 11), infected = $41 \pm 3$ (n = 8), infected + l-NAME = $41 \pm 3$ (n = 8) ($P < 0.001$, 1-way ANOVA), infected + Indo = $41 \pm 3$ (n = 8) ($P < 0.001$, 1-way ANOVA), infected + l-NAME + Indo = $41 \pm 3$ (n = 8) ($P < 0.001$, 1-way ANOVA); Fig. 7).

We next examined the ability of each eicosanoid to abrogate the inhibitory effect of l-NAME on barrier function of infected mucosa (Fig. 8). In the absence of l-NAME, exogenous PG had no effect on barrier function. However, the inhibitory effect of l-NAME on TER was significantly abrogated by exogenous PGE$_2$, but not by carbacyclin (an analog of PGI$_2$; $10^{-6}$ M) or carbocyclic TXA$_2$ ($5 \times 10^{-6}$ or $10^{-6}$ M). Light microscopic examination of l-NAME-treated mucosa rescued with PGE$_2$ demonstrated closure of the paracellular pathway (Fig. 7). Thus, although l-NAME inhibited synthesis of each eicosanoid (PGE$_2$, PGI$_2$, and TXA$_2$), PGE$_2$ was the primary mediator of NO effects on barrier function and closure of paracellular space in infected mucosa.

Expression of NOS isoforms by ileal mucosa in _C. parvum_ infection. In an effort to identify which NOS isoforms were responsible for mediation of PG synthesis and maintenance of barrier function in _C. parvum_ infection, we quantified mRNA expression of each isoform in mucosa from control and infected piglets harvested at the time of our functional studies.
using real-time RT-PCR (n = 5 piglets each; days 3–5 postinfection). Expression of endothelial NOS (eNOS) and nNOS mRNA was not altered by *C. parvum* infection, whereas iNOS mRNA was significantly increased (Fig. 9).

**Constitutive NOS and iNOS promote PG synthesis in *C. parvum* infection.** To determine the contribution of constitutive NOS (cNOS) and iNOS to PG synthesis and barrier function in *C. parvum* infection, sheets of ileal mucosa were incubated in Ussing chambers with NOS isoform-specific inhibitors, and the effect on TER and PG synthesis was measured. As previously shown, inhibition of both iNOS and cNOS activity by L-NAME significantly inhibited PGE₂ synthesis by both infected and control mucosa (Table 2). Greater than 80% of PGE₂ synthesis was NOS dependent in control and infected mucosa. Incubation with L-NIL (30 μM), a selective and irreversible inhibitor of iNOS, had no effect on synthesis of PGE₂ or PGI₂ by control mucosa, as would be expected in the absence of this isoform. In infected mucosa, L-NIL inhibited 45 ± 15% of NOS-dependent PGE₂ synthesis (Fig. 10A) and synthesis of PGI₂ [pg/ml ΔPGI₂ (no. of piglets); infected = 7,013 ± 1,045 (n = 7), infected + L-NIL = 3,874 ± 617 (n = 8) (P < 0.05, 1-way ANOVA)]. In terms of barrier function, inhibition of total NOS or iNOS activity had no effect on TER of control mucosa. In infected mucosa, L-NIL inhibited 50% of NOS-dependent TER, although alone the effect of L-NIL on TER was not statistically significant, even in the presence of high
The serosal bath of control and Cryptosporidium parvum-infected porcine ileal mucosa as measured by ELISA treatment by subtracting 30-min values from 300-min values (measurements negate the effects of baseline differences in eicosanoid synthesis and effects of stripping and mounting of mucosa on the Ussing chambers). The nonselective nitric oxide synthase inhibitor N-nitro-l-arginine methyl ester (l-NAME; 10 nM) significantly inhibited synthesis of each eicosanoid by both control and infected mucosa. *P < 0.05, †P < 0.01, ‡P < 0.001, 1-way ANOVA compared with Ringer solution alone. $P < 0.05 compared with control, ND, not determined.

### DISCUSSION

The present study has demonstrated that NO, elaborated by CNNOS and iNOS, serves as a proximal mediator of PGE2 synthesis and barrier function in *C. parvum* infection (Fig. 11). This conclusion derives from several observations. First, incubation of ileal mucosa with the nonselective NOS inhibitor l-NAME inhibited synthesis of PGE2 in a manner analogous to that of Indo, whereas selective blockade of iNOS using l-NAME blocked ~50% of NOS-dependent (l-NAME-inhibitable) PGE2 synthesis. After blockade of iNOS activity, PGE2 synthesis was reduced to levels seen in uninfected tissue. Second, nonselective inhibition of NOS inhibited TER in a manner equivalent to that of Indo, whereas selective inhibition of iNOS attenuated TER by 50% but alone was insufficient to significantly decrease barrier function. Both nNOS and eNOS can contribute to constitutive NOS synthesis. Inhibitors of nNOS had no effect on PGE2 synthesis or barrier function. Because of inadequate means to selectively block eNOS activity, we could not discern if eNOS inhibition alone could account for the entirety of l-NAME effects on barrier function. However, given the equal contribution of CNNOS and iNOS activity to PGE2 synthesis in the present study and the intermediate effect of l-NAME on TER reduction (Fig. 10), an additive contribution of CNNOS and iNOS to barrier function is plausible. Finally, the loss of barrier function resulting from l-NAME was recovered by exogenous addition of 16,16-dimethyl-PGE2, but not by the synthetic analogs of PGI2 or TXA2.

Increases in transepithelial permeability associated with *C. parvum* are well documented in cell monolayers, in vivo experimental models, and naturally occurring infection (1, 4, 12, 14, 18, 19, 48). In the present study, baseline barrier function, as determined by TER and passive serosal-to-mucosal flux of 22Na+, was significantly impaired by *C. parvum* infection and worsened further by the absence of NO-mediated elaboration of PGE2, NO and reactive nitrogen metabolites, such as peroxynitrite (formed by the reaction of NO with superoxide), have been demonstrated in a variety of experimental models to stimulate COX enzyme activity and PG synthesis (15, 42, 46). We have previously shown that mucosal PG synthesis is increased in *C. parvum*-infected piglets and calves and contributes to barrier function (3, 10). It is likely that the interplay between NOS and COX in the present study involves specific sites of NO production and cellular interactions, insofar as bathing infected mucosa with NO donors fails to recapitulate the effect of endogenous NOS activity on barrier function. Both endogenous NO activity and peroxynitrite formation are diffusion limited, and their effect on PG synthesis has been shown in some studies to depend critically on the identity of the COX isof orm with which they interact (9).

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**Table 2. Concentration of PGE2, 6-keto-PGF1α (the stable metabolite of PGI2), and TXB2 (the stable metabolite of TXA2) in the serosal bath of control and Cryptosporidium parvum-infected porcine ileal mucosa as measured by ELISA**

<table>
<thead>
<tr>
<th></th>
<th>Ringer</th>
<th>Ringer + l-NAME</th>
<th>Ringer + Indo</th>
<th>Ringer + l-NAME + Indo</th>
<th>Ringer</th>
<th>Ringer + l-NAME</th>
<th>Ringer + Indo</th>
<th>Ringer + l-NAME + Indo</th>
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<td></td>
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<tr>
<td>Absolute PGE2, pg/ml</td>
<td>2,079±361 (8)</td>
<td>947±86* (8)</td>
<td>610±120* (8)</td>
<td>451±52* (8)</td>
<td>1,456±346 (8)</td>
<td>313±107† (8)</td>
<td>−13±166‡ (8)</td>
<td>−172±110‡ (8)</td>
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<tr>
<td>Absolute 6-keto-PGF1α, pg/ml</td>
<td>3,187±501§ (14)</td>
<td>1,076±132‡ (15)</td>
<td>649±46‡ (6)</td>
<td>504±52‡ (9)</td>
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<td>322±68§ (8)</td>
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<tr>
<td>Absolute PGE2, pg/ml</td>
<td>6,556±658 (8)</td>
<td>3,231±458‡ (8)</td>
<td>1,085±256‡ (8)</td>
<td>977±198‡ (8)</td>
<td>5,443±647 (8)</td>
<td>2,073±284‡ (8)</td>
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<td>Absolute 6-keto-PGF1α, pg/ml</td>
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<td>ND</td>
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<td>1,753±364‡ (8)</td>
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<tr>
<td>Absolute TXB2, pg/ml</td>
<td>148±24 (7)</td>
<td>92±8* (8)</td>
<td>62±8* (8)</td>
<td>68±10* (8)</td>
<td>94±22 (7)</td>
<td>−7±16† (8)</td>
<td>9±12† (8)</td>
<td>16±9† (8)</td>
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<tr>
<td>Absolute 6-keto-TXB2, pg/ml</td>
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<td>ND</td>
<td>150±21 (7)</td>
<td>40±12‡ (8)</td>
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Values are means ± SE; in parentheses, no. of piglets. TX, thromboxane; Indo, indomethacin (5 × 10⁻⁶ M). Assay samples were obtained 30 and 300 min after each tissue was mounted in an Ussing chamber. Absolute, values measured at 300 min; Δ, absolute change in eicosanoid synthesis in the presence of each treatment by subtracting 30-min values from 300-min values (measurements negate the effects of baseline differences in eicosanoid synthesis and effects of stripping and mounting of mucosa on the Ussing chambers). The nonselective nitric oxide synthase inhibitor N-nitro-l-arginine methyl ester (l-NAME; 10 nM) significantly inhibited synthesis of each eicosanoid by both control and infected mucosa. *P < 0.05, †P < 0.01, ‡P < 0.001, 1-way ANOVA compared with Ringer solution alone. $P < 0.05 compared with control, ND, not determined.
Additionally, broad application of NO has been demonstrated to result in a variety of effects that diminish barrier function of normal epithelial cells in culture (34, 42). The source of elevated PG in C. parvum infection has not been definitively established but may be the result of infiltrating polymorphonuclear neutrophils and macrophages, the products of which have been shown to strongly induce PG synthesis by mesenchymal cells in the lamina propria (2–4, 21). Additionally, C. parvum has been shown to directly activate COX-2 expression and PGE2 synthesis by cultured human intestinal epithelial cells (28).

The mechanism by which PGE2 promotes barrier function of C. parvum-infected mucosa remains to be fully elucidated but appears to involve maintenance of paracellular space closure rather than an effect on epithelial restitution. In mucosa treated with Indo or l-NAME, decline in barrier function was associated with distension of paracellular pathways and expansion of lamina propria consistent with absorption, whereas these effects were reversed with exogenous PGE2. Prior studies of C. parvum infection have demonstrated that PG induce anion (Cl\(-\)/HCO\(_3\)\(^{−}\)) secretion, which promotes withdrawal of Na\(^{+}\) and water from the paracellular spaces of the crypts and inhibit neutral NaCl absorption, resulting in decreased paracellular water absorption by the villus (3). These alterations are attributed to direct effects of PGE2 on the epithelium and indirect effects via PG12 activation of the enteric nervous system (2). Our observation that PG12 did not contribute to barrier function is supported by previous studies in C. parvum-infected piglets demonstrating that inhibition of PG12-activated neurons (using

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Fig. 7. Light microscopic appearance of infected mucosa after incubation in Ussing chambers for 300 min in the presence of indomethacin (Indo; 5 × 10\(^{-6}\) M; A), l-NAME (10 mM; B), and l-NAME + exogenous PGE2 (C). Indo and l-NAME had identical effects on dilation of paracellular pathway of infected mucosa. Addition of exogenous PGE2 reversed the effect of l-NAME on paracellular pathway dilation. Bar = 50 μm.

Fig. 8. TER of C. parvum-infected ileal mucosa after 300 min of incubation in Ussing chambers in the presence of no treatment, l-NAME (10 mM), 16,16-dimethyl-PGE2, the PG12 analog carbacyclin, or the stable thromboxane (TX)A2 analog carbocyclic TXA2 (each 10 × 10\(^{-6}\) M serosal). *\(P < 0.05\), 1-way ANOVA. †\(P = 0.01\), l-NAME vs. l-NAME + PGE2; Student’s paired t-test; n = no. of piglets.

Fig. 9. Effect of C. parvum infection on expression of inducible (iNOS), endothelial (eNOS), and neuronal (nNOS) NOS mRNAs in ileal mucosa. Data represent the degree of difference in mRNA levels adjusted for differences in housekeeping cyclophilin expression among samples. *\(P < 0.05\) vs. control, 1-way ANOVA; n = no. of piglets.
clonidine and somatostatin) does not significantly decrease TER (2).

In the present study, both L-NAME and Indo inhibited TER of infected and not control mucosa. This observation is consistent with prior studies demonstrating that Indo has no effect on TER of uninjured mucosal epithelium but significantly impairs barrier function of leaky epithelium arising as a result of C. parvum infection (3), ischemia (8), or bile salt injury (16). Each of these injuries is associated with a significant increase in mucosal PGE2 synthesis. Although iNOS inhibition appeared to abolish the rise in PGE2 synthesis resulting from C. parvum infection, a reduction in PGE2 synthesis below that seen in uninfected tissues was necessary to significantly impair barrier function. We speculate that basal amounts of PG are not necessary to maintain intercellular space closure in the absence of epithelial injury and have shown that Indo has no effect on NaCl transport or resistance of uninfected tissue (3). In contrast, with ongoing epithelial injury and accelerated restitution, PG effects on NaCl transport may contribute to reestablishment of paracellular space closure and resistance, as has been shown after ischemia and bile salt injury (8, 16).

Results of the present study demonstrate that C. parvum infection leads to induction of iNOS and increased synthesis of NO and PGE2 by parasitized intestinal mucosa. Studies in mice have shown that C. parvum infection is associated with an increase in iNOS expression by the epithelium (31). Although we did not specifically investigate the long-term consequences of iNOS expression in C. parvum infection, prior observations have shown that exposure to high concentrations of NO promotes cytotoxicity and permeability of cultured epithelia (7, 34, 41, 43, 46). These observations suggest a role for iNOS in purging the intestine of the infection. More specifically, NO donors have been shown in vitro to reduce viability of C. parvum sporozoites (30), and iNOS knockout mice or mice treated with NOS inhibitors have more severe intestinal infection and delayed parasite clearance (30, 31). Nevertheless, the
role of epithelial vs. subepithelial iNOS in mediating these effects remains unclear. Although iNOS may promote elimination of infected enterocytes, overall epithelial barrier function appears to be promoted by the paracellular effects of NO-mediated PGE2 synthesis.

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