Enteroinvasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells

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Enteroinvasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells. Am. J. Physiol. 38: G564–G571, 1998.—In these studies, we investigated whether bacterial infection of human colon epithelial cells is a sufficient stimulus to upregulate epithelial cell expression of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production. Human colon epithelial cells (Caco-2 and HT-29) rapidly upregulated iNOS mRNA and nitric oxide (NO) production. Enteroinvasive bacteria, including E. coli, Salmonella dublin, or Shigella flexneri but not after infection with noninvasive E. coli or an invasion-deficient mutant of S. dublin. Bacterial infection in the absence of added cytokines was as potent or more potent a stimulus of iNOS expression and NO production as stimulation of cells with combinations of cytokines known to strongly upregulate this epithelial cell response. Enteroinvasive E. coli increased epithelial NO production to a greater extent than S. dublin, although S. dublin was a stronger stimulus of epithelial cell interleukin-8 (IL-8) production. After enteroinvasive E. coli infection of polarized epithelial cell monolayers, nitrite, a stable NO end product, was released predominantly into the apical compartment early after infection, whereas IL-8 was released in parallel into the basolateral compartment. These studies suggest NO and/or its redox products are an important component of the intestinal epithelial cell response to microbial infection.

Intestinal inflammation; microbial pathogenesis; host response; polarized epithelial cells; Salmonella; Escherichia coli

THE SINGLE LAYER of epithelial cells that lines the intestinal mucosa is the initial site of interaction between the host and enteroinvasive microbial pathogens. Intestinal epithelial cells respond to bacterial invasion by upregulating the expression of an inflammatory gene program (25). Characteristic features of this program include the increased production of chemokines that can act as early signals to activate an acute mucosal inflammatory response (8, 24, 53) and the increased capacity of epithelial cells to produce prostanoids, which in turn can alter epithelial cell secretory functions (10).

Nitric oxide (NO) is generated by the conversion of l-arginine to l-citrulline by NO synthase (NOS), which exists in three isoforms, each encoded by a separate gene (39, 40). The expression of inducible NOS (iNOS, encoded by NOS2) is regulated in various cell types and can be increased by stimulation of cells with several cytokines or with bacterial lipopolysaccharide (LPS) (39, 40). iNOS can also be expressed constitutively, as has been shown for human lung epithelial cells (2, 15) and murine ileal epithelial cells (16). Human colon epithelial cell lines are known to upregulate iNOS expression and the production of NO and its redox products (NOx) in response to stimulation with a combination of interferon-γ (IFN-γ) and interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α) (22, 27, 30, 43). Consistent with the ability of these cytokines to upregulate intestinal epithelial cell iNOS expression, increased iNOS expression has been reported in colon epithelial cells of patients with inflammatory bowel disease in areas of acute inflammation (47). In contrast to iNOS, neuronal NOS (nNOS, encoded by NOS1) and endothelial NOS (eNOS, encoded by NOS3) are constitutively expressed, although recent studies indicate their expression can also be regulated (36, 39, 40).

Increased iNOS expression was recently noted in the surface colon epithelium of patients during acute Shigella colitis (19), and epithelial cells from rats challenged in vivo with LPS from Escherichia coli manifested increased iNOS activity (48). However, Salmonella dublin, an enteroinvasive bacteria that is known to be a potent agonist of the intestinal epithelial cell proinflammatory gene program (8, 10, 17, 24), did not upregulate iNOS expression or NO production in human colon epithelial cell lines in the absence of costimulation of those cells with IFN-γ (45). The latter findings suggest that bacterial infection alone is not a sufficient stimulus to upregulate epithelial iNOS expression and NO production in the absence of concurrent mucosal inflammation and the associated increased production of cytokines such as IFN-γ and IL-1 or TNF-α.

NO can mediate an array of physiological effects in the intestine that are relevant to neurotransmission, regulation of vascular functions, and epithelial barrier integrity (1). Moreover, NO produced by intestinal epithelial cells could play a role in the host’s response to enteric microbial pathogens (12, 31, 38). In this regard, NO produced by macrophages was shown to be cytostatic or cytotoxic for a broad spectrum of microbial pathogens, including intracellular parasites (e.g., Leishmania major, Schistosoma mansoni, Trypanosoma cruzi, Toxoplasma gondii) (20, 21, 29, 46) and bacteria (e.g., Mycobacterium tuberculosis) (33), and NO inhibited replication of herpes simplex virus and coxsackievirus in vitro (5, 55). Furthermore, NO can modulate the agonist-stimulated release of cytokines, including IL-6 and TNF-α, by macrophages in vitro (7). Nonetheless, mice deficient for either of the three NOS isoforms are generally healthy, although nNOS-deficient mice de-
develop pyloric stenosis and eNOS-deficient mice have elevated arterial blood pressures (18). However, iNOS-deficient mice are highly susceptible to infections with Leishmania major and Listeria monocytogenes (32, 51) and have increased severity of colitis inflammation in experimental models (35).

The present study demonstrates that infection of human colon epithelial cells with enteroinvasive bacteria activates epithelial cell iNOS expression and NO production in the absence of IFN-γ. Moreover, as shown herein, enteroinvasive E. coli are a stronger stimulus for the upregulated expression of iNOS and NO production in human colon epithelial cell lines than S. dublin and as potent or more potent a stimulus than known cytokine agonists. Consistent with a role for epithelial cell-derived NO in intestinal antimicrobial defense, nitrite, a stable end product of NO, is shown to be released predominantly at the apical epithelial cell surface after apical bacterial infection of polarized Caco-2 epithelial cells.

MATERIALS AND METHODS

Colon epithelial cell lines and cell cultures. HT-29 [American Type Culture Collection (ATCC) HTB 38] human colonic epithelial cells were obtained from ATCC (Manassas, VA), and Caco-2 cells were obtained from S. Tzipori (Tufts University). Cells were grown in RPMI 1640 or DMEM supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 10 mM HEPES, 50 U/ml penicillin, and 50 µg/ml streptomycin. T84 human colonic epithelial cells were grown in DMEM-F12 with 5% newborn calf serum, as described previously (8, 24). Cells were maintained in media without antibiotics for 4–6 days before infection.

Colon epithelial cells (1 × 10⁶) were seeded in a 1-ml volume into six-well Costar tissue culture plates and grown to ~90% confluence before use. For polarized monolayers, Caco-2 cells were grown on collagen-coated microporous filter supports (0.4-µm pore size, 24-mm diameter) in Transwell cultures (Costar, Cambridge, MA) for 10–14 days. During this time, culture medium (RPMI 1640 or DMEM supplemented with 0.5% FCS, 2 mM l-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin) was changed every 2 days. Monolayers were kept in medium without antibiotics for 4–6 days before infection. The formation of tight junctions was assessed functionally by measuring electrical resistance across the monolayers with the use of a Millicell electrical resistance system (Millipore, Bedford, MA). The electrical resistance of uninfected monolayers ranged from 300 to 400 Ω·cm² after subtraction of resistance across a cell-free filter.

Cytokines, other reagents, and bacteria. The following cytokines were used in these studies: recombinant human (rh) TNF-α (R&D Systems, Minneapolis, MN), rhL-1β (Pepolex, Natlack, MA), and rhIFN-γ (Biosource International, Camarillo, CA). LPS (from E. coli serotype O111:B4) purchased from Sigma Chemical (St. Louis, MO), N⁵-nitro-L-arginine methyl ester (L-NAME) was purchased from ICN (Biosource International, Camarillo, CA), and N⁵-nitro-L-arginine (L-NNA) was obtained from Sigma. Enteroinvasive E. coli strain O29:NM was obtained from ATCC (43892). S. dublin lane strain, an isogenic invA mutant of S. dublin, and Shigella flexneri were provided by Dr. J. Fierer [University of California, San Diego (UCSD)], and nonpathogenic E. coli DH5α were purchased from GIBCO BRL (Gaithersburg, MD).

Infection protocol. Bacteria were grown at 37°C in tryptic soy broth overnight. Cell cultures were infected as described previously (8, 10, 17). Briefly, bacteria were added in a 1-ml volume to each well and incubated for 1 h to allow bacterial entry to occur. Monolayers were washed three times with PBS to remove extracellular bacteria, and the cultures were further incubated for 3–24 h in the presence of 50 µg/ml gentamicin to kill remaining extracellular bacteria (8). After infection, culture media were removed and centrifuged for 15 min at 12,000 g, and supernatants were stored at 4°C until use.

Nitrite and nitrate assays. NO is rapidly converted into the stable end products nitrate and nitrate. Nitrite in culture supernatants was measured by the Griess reaction (14). Briefly, 100 µl of culture supernatant were mixed with an equal volume of Griess reagent (0.5% sulfanilamide, 2.5% H₃PO₄, and 0.05% naphthylethylene diamine in H₂O) and incubated for 10 min at room temperature. Absorbance was assayed at 550 nm and compared with a standard curve obtained using sodium nitrate. The assay was sensitive to 1 µM nitrite. Nitrate was determined using the Griess reaction after the conversion of nitrate to nitrite by nitrate reductase. Those experiments included a standard curve constructed using known concentrations of sodium nitrate. In brief, 50 µl of culture medium were incubated for 1 h with 39 µl MOPS-EDTA (50 mM, pH 7.0), 10 µl NADH (10 µM), and 5 µl of 5 U/ml nitrate reductase from soybean seedlings (Sigma). To obtain ratios of nitrite to nitrate produced after bacterial infection, cells were grown in DMEM, which contains only small amounts of nitrate (<1 µM), whereas RPMI 1640 medium contains high concentrations of nitrate (~600 µM).

Quantitative RT-PCR analysis. Total cellular RNA was extracted from colon epithelial cells with the use of TRIzol reagent (GIBCO BRL), and RNA integrity was confirmed by electrophoresis on 1% agarose gels and ethidium bromide staining. iNOS mRNA levels were quantitated by RT-PCR using standard RNA (10, 24, 42, 53). Standard RNA was generated by in vitro transcription of a construct, TLMNOS2, using T7 RNA polymerase as described previously (10, 42). TLMNOS2 was generated using methods previously described (10, 42) and encodes 5' and 3' priming sites for iNOS. Primers for human iNOS were obtained from Clontech (Palo Alto, CA) (sense primer, 5'-CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG-3'; antisense primer 5'-GCT TGT TAG GAG GTA TAA GC-3'). The size of the PCR product for standard and target RNAs was 356 and 259 bp, respectively. Primers for eNOS and nNOS were as follows: sense primer, 5'-GTG ATG GCG AAG CGA GTG AAG-3' and antisense primer, 5'-CCG AGC CCC AAC ACA CAG AAC-3'. These eNOS primers yielded a PCR product of 422 bp. Primers for nNOS were purchased from Clontech (Palo Alto, CA) and yielded a PCR product of 471 bp. Primers for β-actin were as described previously (24).

PCR amplification consisted of 35 cycles of 45 s of denaturation at 94°C, 45 s of annealing at 60°C, and 2 min of extension at 72°C. Before PCR and addition of Taq polymerase (Stratagene, La Jolla, CA), the samples were heated to 95°C for 5 min to increase specificity of the amplifications (“hot start”). For negative controls, RNA was omitted from the reverse transcription mixture and cDNA was omitted from the PCR reaction. Positive controls for eNOS and nNOS used RNA isolated from human umbilical cord endothelial cells (HUVEC-C; ATCC CRL 1730) and 1321N1 human astrocytoma cells (gift of Dr. J. Brown, UCSD), respectively. After amplification, PCR products were separated on a 1% agarose gel (Ultrapure; GIBCO BRL) and visualized by ethidium bromide staining. Photographs of the gels were taken, and the density of the bands was used to calculate the number of cellular RNA transcripts as described previously (24).
method can detect \(10^2\) mRNA transcripts per microgram of cellular RNA.

Analysis of iNOS expression by immunoblot. Bacteria-infected and control monolayers were assayed for iNOS expression by immunoblot analysis. Briefly, cells were washed with PBS (pH 7.4) and then harvested in lysis buffer containing protease inhibitors (150 mM NaCl, 20 mM Tris, pH 7.4, 1% Triton X-100, 10 µg/ml phenylmethylsulfonyl fluoride, 1,000 U/ml aprotinin, and 25 µg/ml leupeptin). Protein content was measured by the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein (20 µg) from each cell lysate were electrophoresed on 8% SDS-polyacrylamide gels, after which the protein was transferred to a nitrocellulose membrane (Schleicher & Schuell, Kenee, NH). Membranes were incubated with rabbit anti-human iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or control rabbit IgG, followed by donkey anti-rabbit IgG linked to horseradish peroxidase, and developed using enhanced chemiluminescence Western blotting detection reagents (Amersham Life Science, Arlington Heights, IL) and exposure to X-ray film (XAR5; Eastman Kodak, Rochester, NY).

**RESULTS**

Increased nitrate production by colon epithelial cells in response to infection with enteroinvasive E. coli or Salmonella. NO production, as assessed by assaying its stable end product nitrite, increased greater than 100-fold by 4–6 h after infection of Caco-2 cells with enteroinvasive E. coli and remained increased above baseline for the 24-h culture period (Fig. 1). The greatest increase in nitrite production in response to enteroinvasive E. coli infection was observed between 4 and 8 h after infection. Thus the differences between the cumulative nitrite levels at the beginning and end of different 4-h periods after infection with enteroinvasive E. coli were 36 µM for the period 0–4 h after infection, 55 µM for the 4- to 8-h period postinfection, 18.5 µM for the 8- to 12-h period postinfection, and 3.7 µM for the 20- to 24-h period postinfection. NO production also significantly increased after infection of Caco-2 cells with the invasive bacterial pathogen S. dublin, albeit to a lesser extent (Fig. 1). In contrast, cumulative nitrite production in uninfected control cultures remained below the detection limit of the assay (i.e., <1 µM) over the 24-h culture period. For comparison, after stimulation of Caco-2 cells for 24 h with combinations of cytokines (i.e., 10 ng/ml each of IL-1β, IFN-γ, and TNF-α in combination, or 10 ng/ml each of IL-1β and IFN-γ), nitrite levels in the supernatants were as much as 17-fold lower than after infection of parallel cultures with enteroinvasive E. coli (nitrite levels in response to these cytokine combinations ranged from 8.3 to 58.8 µM; mean 28.6 ± 6.3 µM, n = 9). Furthermore, consistent with prior studies of cytokine-stimulated DLD-1 colon epithelial cells (43), the ratio of nitrate to nitrite produced after bacterial infection or cytokine stimulation of Caco-2 cells was ~10:1.

To determine if NO production was increased in response to bacterial infection in other human colon epithelial cell lines, HT-29 and T84 cells were infected with enteroinvasive E. coli or S. dublin. As with Caco-2 cells, nitrite levels were significantly increased in HT-29 cells infected with enteroinvasive E. coli or S. dublin [cumulative nitrite release was 286 ± 43 µM at 24 h after infection with enteroinvasive E. coli (2.5 × 10^9 bacteria/ml) and 50 ± 5 µM at 24 h after infection with S. dublin (2.5 × 10^8 bacteria/ml), whereas <1 µM nitrite was present in supernatants from control cultures; values are means ± SE of 3 or more experiments]. In contrast, T84 cells released only low levels of nitrite (<2 µM) after infection with enteroinvasive E. coli or S. dublin.

The magnitude of increased NO production after infection of Caco-2 cells with enteroinvasive E. coli depended on the bacterial inoculum, as shown in Table 1. Furthermore, neither a noninvasive E. coli (strain DH5α), an isogenic invA mutant of S. dublin that is 100-fold less invasive than wild-type S. dublin (8), nor addition of bacterial LPS to Caco-2 cells resulted in a substantial increase in NO production. Because patients with Shigella colitis manifest increased iNOS expression in surface colon epithelial cells (19), Caco-2 cells were infected with S. flexneri in a separate experiment. As shown in Table 1, S. flexneri increased NO production by Caco-2 cells to an extent similar to that of enteroinvasive E. coli.

Bacterial infection increases expression of epithelial iNOS. To determine whether increased NO release was paralleled by increased iNOS production, iNOS expression was assessed by immunoblot analysis at 3 and 24 h after infection of Caco-2 cells with enteroinvasive E. coli or S. dublin. As shown in Fig. 2 for enteroinvasive E. coli-infected cells, iNOS levels were upregulated at 3 and 24 h postinfection, with the greater increase at 3 h. Similarly, S. dublin infection of Caco-2 cells also increased iNOS levels at 3 and 24 h after infection (data not shown).
6-well plates were either infected with enteroinvasive *Escherichia coli*. Measurable levels of nitrite alone in culture media in the absence of epithelial cells did not result in the Griess reaction. Incubation of comparable numbers of bacteria *S. dublin*, stimulated with bacterial lipopolysaccharide (LPS), *S. flexneri*, or an invasion-deficient isogenic (serotype O29:NM), a nonpathogenic *E. coli* for *S. dublin*, SB133, stimulated with bacterial lipopolysaccharide (LPS) (20 μg/ml), or not infected or stimulated (None). Supernatants were collected after 24 h, and nitrite concentrations were determined by the Griess reaction. Incubation of comparable numbers of bacteria alone in culture media in the absence of epithelial cells did not result in measurable levels of nitrite.

Bacterial infection upregulates iNOS mRNA levels in Caco-2 cells. To determine whether increased iNOS levels in Caco-2 cells were paralleled by increased iNOS mRNA expression, Caco-2 cells were infected with enteroinvasive *E. coli*, and the kinetics of iNOS mRNA expression were determined by quantitative RT-PCR analysis. As shown in Fig. 3, iNOS mRNA levels increased within 1 h postinfection and reached a maximal increase of ~100-fold at 8 h postinfection. Subsequently, iNOS mRNA levels decreased but remained elevated 10-fold above controls at 24 h postinfection. This time course parallels the increases in iNOS protein levels (see Fig. 2). Neither Caco-2 nor HT-29 cells constitutively expressed significant levels of mRNA for nNOS or eNOS, and neither nNOS nor eNOS mRNAs were upregulated by bacterial infection of Caco-2 or HT-29 cells, as determined by RT-PCR (Fig. 4 and data not shown).

Nitrite production by Caco-2 cells is blocked by NOS inhibitors. The arginine analog L-NAME is a competitive inhibitor of NOS. To demonstrate that increased NO production in colon epithelial cells in response to bacterial infection was NOS dependent, Caco-2 cells infected with enteroinvasive *E. coli* were incubated with increasing concentrations of L-NAME. As shown in Fig. 5, increasing doses of L-NAME inhibited epithelial cell NO production in a dose-dependent manner. Similarly, NO production in infected cells was inhibited by the NOS inhibitor aminoguanidine (100 μM aminoguanidine inhibited NO production by 77.6%).

Nitrite production is predominantly apical early after infection of polarized Caco-2 cell monolayers. Intestinal epithelial cells are structurally and functionally polarized in vivo. The Caco-2 cell line studied herein can be used to assess host innate immune responses to bacterial infection of intestinal epithelial cells.

Table 1. Increased nitrite production by bacteria-infected Caco-2 cells is dependent on the bacterial strain and the bacterial inoculum.

<table>
<thead>
<tr>
<th>Additions to Culture</th>
<th>Bacterial Inoculum/Well</th>
<th>Nitrite Produced, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O29:NM</td>
<td>2.5 × 10⁸</td>
<td>201.8 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>2.5 × 10⁹</td>
<td>103.6 ± 1.3</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>2.5 × 10⁸</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>5.0 × 10⁹</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td><em>S. dublin</em></td>
<td>2.5 × 10⁹</td>
<td>37.5 ± 15.0</td>
</tr>
<tr>
<td><em>S. dublin</em> invA</td>
<td>5.0 × 10⁹</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td><em>S. flexneri</em></td>
<td>5.0 × 10⁹</td>
<td>153.0</td>
</tr>
<tr>
<td>LPS</td>
<td>&lt; 1.0</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>&lt; 1.0</td>
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</table>

Values are means ± SE of 3 or more repeated experiments. Values for *Shigella flexneri* are from a single experiment. Caco-2 cells in 6-well plates were either infected with enteroinvasive *Escherichia coli* (serotype O29:NM), a nonpathogenic *E. coli* (DH5α), wild-type *Salmonella dublin*, or an invasion-deficient isogenic invA mutant of *S. dublin*, SB133, stimulated with bacterial lipopolysaccharide (LPS) (20 μg/ml), or not infected or stimulated (None). Supernatants were collected after 24 h, and nitrite concentrations were determined by the Griess reaction. Incubation of comparable numbers of bacteria alone in culture media in the absence of epithelial cells did not result in measurable levels of nitrite.

### Table 2. Produced, µM

<table>
<thead>
<tr>
<th>Bacterial Inoculum/Well</th>
<th>Nitrite Produced, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>6.0</td>
</tr>
<tr>
<td>1.0</td>
<td>6.0</td>
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<tr>
<td>6.0</td>
<td>15.0</td>
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</table>

Fig. 2. Bacterial infection increases iNOS levels in Caco-2 cells. Monolayers of Caco-2 cells were infected with enteroinvasive *E. coli* O29:NM (5 × 10⁸ bacteria/ml) or left uninfected (controls). Cell lysates were prepared 3 or 24 h later, size fractionated, and blotted onto a nitrocellulose membrane, and iNOS was detected using a specific antibody and enhanced chemiluminescence as described in MATERIALS AND METHODS. Representative examples of X-ray films are shown. A major band representing iNOS was present at 130 kDa. The second lower molecular weight band may represent a breakdown product of iNOS or the product of an alternatively spliced iNOS message (11).

Fig. 3. Bacterial infection upregulates iNOS mRNA levels. Caco-2 cells grown to 90% confluence in 6-well plates were infected with enteroinvasive *E. coli* (5 × 10⁹ bacteria/well) (●) or not infected (○). Cells were harvested at indicated times, and iNOS mRNA levels were determined by RT-PCR. Data are from a representative experiment. Similar data were obtained in 3 repeated experiments.

Fig. 4. Bacterial infection does not induce nNOS and eNOS mRNA expression. Caco-2 cells were grown and infected as described in Fig. 3 legend. RNA was harvested from infected and uninfected cells 24 h later, and mRNA expression for eNOS (lanes 1–5), nNOS (lanes 6–10), and β-actin (lanes 11–16) was assessed by RT-PCR using 35 cycles of amplification. Lanes 1, 6, and 11 are from uninfected Caco-2 cells; lanes 2, 7, and 12 are from *S. dublin*-infected Caco-2 cells; lanes 3, 8, and 13 are from Caco-2 cells infected with enteroinvasive *E. coli*, lanes 4 and 15 are from uninfected human umbilical endothelial cells, and lanes 9 and 14 are from uninfected human astrocytoma cells that were used as positive controls for eNOS and nNOS, respectively. Lanes 5, 10, and 16 contain no RNA. Similar results were obtained when infected and uninfected cells were harvested at earlier time points (6, 12, and 18 h). Faint bands for nNOS and eNOS could be detected in uninfected Caco-2 cells, and a faint band for nNOS was seen in HT-29 cells, but only after 40 cycles of amplification, and these bands were not further increased in enteroinvasive *E. coli*-infected cells (not shown).
grown in Transwell chambers as polarized monolayers that form tight junctions, as assessed by transepithelial electrical resistance and several other parameters (28). We have shown previously that the neutrophil chemoattractant IL-8 is mainly secreted from the basolateral surface after infection of polarized monolayers of T84 and Caco-2 cells with enteroinvasive bacteria and Cryptosporidium parvum, respectively (8, 28). Unlike IL-8, NO is a small, freely diffusible molecule that would be predicted to be released from infected epithelial cells both apically and basolaterally. However, as shown in Fig. 6, in the early period (12–24 h) after infection of polarized Caco-2 monolayers with enteroinvasive E. coli, increased nitrite levels were found predominantly in the apical compartment. By 48–72 h postinfection, nitrite levels were approximately equal in the apical and basolateral compartments. In contrast, IL-8 was secreted almost exclusively into the basolateral compartment throughout the 72-h culture period.

DISCUSSION

In the present studies, we investigated whether upregulation of iNOS and NO production during bacterial infection of the intestinal epithelium can occur as a direct epithelial cell response to bacterial infection. The alternative possibility is that upregulated iNOS expression during the course of bacterial infection simply reflects stimulation of intestinal epithelial cells with IFN-γ and IL-1, which are released during mucosal infection. To address this question, human colon epithelial cell lines, which do not produce IFN-γ and which were free of contaminating cells, were infected with three different invasive bacteria, enteroinvasive E. coli, S. dublin, or S. flexneri. Our results show that human colon epithelial cell lines can upregulate the expression of iNOS and the production of NO in direct response to infection with enteroinvasive bacteria, in the absence of added cytokines.

Upregulated expression of iNOS mRNA in bacteria-infected cultures was seen within 1 h postinfection, was maximal by 8 h postinfection, and remained increased above control levels for at least 24 h. In parallel, epithelial cell NO production was increased and remained elevated for at least 24–72 h, as assessed by release into the culture media of nitrite and nitrate, the stable end products of NO. The kinetics of increased iNOS expression and NO production resemble those for upregulated prostaglandin H synthase-2 (PGHS-2) (the inducible form of cyclooxygenase) mRNA expression and PGE2 production and ENA-78 mRNA expression after bacterial infection (10, 53). However, they differ from those of several chemokines (e.g., IL-8, GRO) that are also upregulated in human colon epithelial cells in response to bacterial invasion but reach maximal levels of expression more rapidly (i.e., within 2–3 h) (8, 53). Experiments using a nonpathogenic E. coli or an invasion-defective isogenic mutant of S. dublin suggest a requirement for bacterial invasion to upregulate iNOS expression and NO release, as is also the case for the
activation of epithelial chemokines and other proinflammatory genes, as well as PGHS-2 and intercellular adhesion molecule-1 (ICAM-1) (8, 10, 17, 24).

In the first 24 h after apical infection of polarized monolayers of Caco-2 cells in Transwell cultures, the stable end product of NO, nitrite, accumulated mainly in the apical compartment. The apical accumulation of nitrite stands in marked contrast to the polarized basolateral release of the neutrophil chemoattractant IL-8 in the same experiment and from T84 and Caco-2 cells infected with Salmonella or C. parvum, respectively, in previous studies (8, 28). It is compatible, however, with recent in vivo studies in humans and mice that demonstrated a predominant localization of iNOS in the apical cytoplasm of intestinal epithelial cells (16, 19). However, it is not known whether increased nitrite levels in the apical compartment reflect 1) NO diffusion across the apical membrane, with its subsequent conversion to nitrite outside the cell, 2) the apical release of nitrite formed from NO within epithelial cells, or 3) the less likely possibility that nitrite is selectively transported from the basolateral to the apical compartment. If either of the latter two possibilities is the case, our results can be interpreted to suggest the presence of an apical membrane transporter for nitrite, perhaps analogous to a cotransporter recently noted in mammalian cells for nitrate (4), or an apical anion channel for nitrite.

Infection with enteroinvasive E. coli was a significantly more potent stimulus of increased NO production in the cell lines tested than S. dublin infection, although S. dublin was a more potent stimulus in the same cells for increased IL-8 production (8, 24). A recent report concluded that infection with invasive bacteria upregulated iNOS expression and NO production in human colon intestinal epithelial cells only in the presence of added IFN-γ as a costimulus (45), but those studies relied solely on the use of S. dublin, which is shown herein to be a relatively weak inducer of this response. Finally, we note that NO production increased minimally, if at all, in bacteria-infected T84 cells. It is possible that infection with invasive bacteria is not a good stimulus for increased NO production in T84 cells, although chemokine production and ICAM-1 expression are strongly increased in these cells by the same stimuli (8, 17, 24). Alternatively, T84 cells may not produce NO under any conditions, possibly because they lack sufficient NOS expression. In support of the latter possibility, T84 cells are generally considered to be representative of human intestinal crypt epithelium (34), which did not express iNOS during the course of Shigella infection in vivo (19).

Enteroinvasive bacteria (e.g., enteroinvasive E. coli, Shigella, and Salmonella) use different mechanisms for entering host cells (13). Moreover, after invading epithelial cells, these bacteria have different intracellular lifestyles. For example, Shigella move freely in the cytoplasm, whereas Salmonella reside in intracellular vesicles (13). Nonetheless, each of these enteroinvasive bacteria can upregulate the expression and production of epithelial cell proinflammatory genes and products, including C-X-C and C-C chemokines (8, 24, 53), PGHS-2 and prostaglandins E2 and F2α (10), the adhesion molecule ICAM-1 (17), and, as shown herein, the production of NO. Enteroinvasive bacteria can also activate specific receptor-independent processes in intestinal epithelial cells. For example, infection of human colon epithelial cells with Salmonella, but not other enteroinvasive bacteria, increased D-myo-inositol-1,4,5,6-tetrakisphosphate production in these epithelial cells, which in turn promoted mechanisms resulting in increased epithelial cell Cl− secretion (9).

Enteroinvasive E. coli were more potent than S. dublin in activating epithelial NO production, although both bacteria can invade the human colon epithelial cell lines used herein to a similar extent (10, 24). Such findings suggest qualitative and/or quantitative differences in the activation of intracellular signaling pathways and subsequent gene transcription by enteroinvasive E. coli and S. dublin. Thus stimulation of human colon epithelial cells with IL-1 alone can activate the nuclear factor-κB (NF-κB) transcription factor complex and its target gene, IL-8 (23), whereas agonist-stimulated upregulation of iNOS expression requires stimulation of the same cells with IL-1 and IFN-γ in combination (30, 43). This indicates that the upregulation of iNOS expression requires the activation of additional intracellular signals and additional transcription factors besides the NF-κB dimers important in IL-8 activation. Our findings suggest that different enteroinvasive bacteria can differentially activate intracellular signaling pathways that are known to be activated by specific agonists such as IL-1 and IFN-γ.

The role of epithelial cell-derived NO in the pathogenesis of enteric infections with invasive bacteria is not known at present. NO could have antimicrobial functions, but it could also promote the spread of the infection. In support of the latter, NO has been shown to dilate tight junctions between epithelial cells in polarized intestinal epithelial monolayers (44, 49, 50), although the converse can also be the case (26). Moreover, NO produced by epithelial cells can interact with superoxide to generate peroxynitrite, which in turn can damage host cells (41, 47). However, the preponderance of evidence suggests an antimicrobial role for NO in the intestine. Thus NO and/or its redox products can be cytostatic for enteroinvasive bacteria, including Salmonella (6, 12), and may decrease microbial entry into epithelial cells by increasing the epithelial protective barrier through the release of epithelial mucus (3).

Bacterial clearance from the intestinal lumen may also be increased by NO through its ability to induce intestinal fluid secretion (37, 52). Furthermore, consistent with a role for NO in antimicrobial host defense at the apical surface of intestinal epithelial cells, NO is more stable at low O2 tensions such as those present in the colonic lumen (54), and we found increased levels of stable NO end products mainly in the apical compartment of polarized epithelial monolayers.

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