Nitric oxide inhibits enterocyte migration through activation of RhoA-GTPase in a SHP-2-dependent manner

Selma Cetin,1 Cynthia L. Leaphart,1 Jun Li,1 Irene Ischenko,2 Michael Hayman,2 Jeffrey Upperman,1 Ruben Zamora,1 Simon Watkins,3,4 Henri R. Ford,1 James Wang,5 and David J. Hackam1,4,5

1Division of Pediatric Surgery, Department of Surgery, Children’s Hospital of Pittsburgh, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania; 2Department of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, New York; 3Center for Biologic Imaging, 4Department of Cell Biology and Physiology, and 5Department of Orthopedic Surgery, Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania

Submitted 12 August 2006; accepted in final form 30 January 2007

Nitric oxide inhibits enterocyte migration through activation of RhoA-GTPase in a SHP-2-dependent manner. Am J Physiol Gastrointest Liver Physiol 292: G1347–G1358, 2007. First published February 1, 2007; doi:10.1152/ajpgi.00375.2006.—Diseases of intestinal inflammation like necrotizing enterocolitis (NEC) are associated with impaired epithelial barrier integrity and the sustained release of intestinal nitric oxide (NO). NO modifies the cytoskeletal regulator RhoA-GTPase, suggesting that NO could affect barrier healing by inhibiting intestinal restitution. We now hypothesize that NO inhibits enterocyte migration through activation of RhoA-GTPase and sought to determine the pathways involved. The induction of NEC was associated with increased enterocyte NO release and impaired migration of bromodeoxyuridine-labeled enterocytes from terminal ileal crypts to villus tips. In IEC-6 enterocytes, NO significantly inhibited enterocyte migration and activated RhoA-GTPase while increasing the formation of stress fibers. In parallel, exposure of IEC-6 cells to NO increased the phosphorylation of focal adhesion kinase (pFAK) and caused a striking increase in cell-matrix adhesiveness, suggesting a mechanism by which NO could impair enterocyte migration. NEC was associated with increased expression of pFAK in the terminal ileal mucosa of wild-type mice and a corresponding increase in disease severity compared with inducible NO synthase knockout mice, confirming the dependence of NO for FAK phosphorylation in vivo and its role in the pathogenesis of NEC. Strikingly, inhibition of the protein tyrosine phosphatase (SHP-2) in IEC-6 cells prevented the activation of RhoA by NO, restored focal adhesions, and reversed the inhibitory effects of NO on enterocyte migration. These data indicate that NO impairs mucosal healing by inhibiting enterocyte migration through activation of RhoA in a SHP-2-dependent manner and support a possible role for SHP-2 as a therapeutic target in diseases of intestinal inflammation like NEC.

intestinal inflammation; restitution; epithelial barrier; necrotizing enterocolitis; bacterial translocation

NECROTIZINGENTEROCOLITIS (NEC) is the leading cause of death from gastrointestinal disease in neonates and is associated with a disruption in the protective intestinal barrier (2, 23). Barrier injury allows for the translocation of enteric bacteria, causing activation of macrophages in the lamina propria and the initiation of a systemic inflammatory response (17). A critical mediator of the inflammatory response in the pathogenesis of NEC is nitric oxide (NO), a small, highly reactive gas that acts as a signaling molecule and forms reactive intermediates that alter cell function (68). During NEC, NO is released by the inflammatory cells that are recruited to the sites of mucosal disruption (32, 53) and by the enterocytes themselves in response to cytokine induction of the enzyme inducible NO synthase (iNOS) (9). High levels of exogenous NO have been shown to exert cytotoxic effects on the intestine that worsen the mucosal injury (49). Although an increase in NO is clearly associated with the development of NEC (19), the mechanisms by which NO release contributes to the pathogenesis of NEC remain incompletely understood.

Healing of the intestinal mucosa occurs through the process of restitution, in which healthy enterocytes migrate to sites of injury to bridge the mucosal defect. Studies from our laboratory have demonstrated that intestinal restitution is significantly impaired in experimental NEC (10). We and others (58, 59) have also shown that enterocyte migration is regulated in part by the Rho family of small-molecular-weight GTPases, including Rho, Rac, and Cdc42, which control the dynamic changes in cytoskeletal structure that allow movement to occur. During NEC, bacterial lipopolysaccharide leads to activation of RhoA, which results in enhanced cell-matrix adhesive-ness and impaired migration (10). We have also demonstrated (51) that the release of bacterial lipopolysaccharide leads to an increase in enterocyte integrin expression and function, which in part explains the increase in adhesion of the enterocyte to the underlying matrix and the block in migration. Although enterocyte migration plays a critical role in the healing from the intestinal injury that characterizes NEC, a link between the release of NO in the pathogenesis of NEC and the impairment in enterocyte migration remains largely unexplored.

One of the potential molecular links between NO and the cytoskeletal changes required for enterocyte migration to occur is the NH2-terminal src homology-2 (SH2)-containing protein tyrosine phosphatase (SHP-2). SHP-2 activity has been shown to be involved in the regulation of the cytoskeleton of various cells (71), and NO has been shown to activate SHP-2 in vascular smooth muscle cells (11). NO has also been shown to stimulate the tyrosine phosphorylation of focal adhesion kinase (pFAK) in fibroblasts through undefined mechanisms, suggesting that a link may exist between NO release and cell-matrix adhesion formation (40). Given that SHP-2 signaling occurs within the intestine (64), the above-mentioned studies raise the intriguing possibility that SHP-2 could provide a link between...
NO release and the impaired enterocyte migration observed in NEC.

We now hypothesize that NO impairs enterocyte migration and that this effect occurs through a SHP-2-mediated activation of the Rho-GTPase-FAK signaling pathway. We now show that NEC is associated with mucosal release of NO, that exogenous NO inhibits enterocyte migration in a dose-dependent manner both in vitro and in vivo. We further demonstrate that inactivation of SHP-2 by using dominant negative constructs reverses the effects of NO on Rho-GTPase signaling and enterocyte migration, suggesting that a novel RhoA-SHP-2-FAK pathway becomes activated on NO treatment, leading to an inhibition of enterocyte migration.

MATERIALS AND METHODS

Cell culture, transfection, and treatment. IEC-6 cells were obtained from the American Type Culture Collection and were maintained as described (49). Where indicated, cells were treated with the NO donors S-nitroso-N-acetylpenicillamine (SNAP) or diethylamine-triamine-NONOate (detaNONOate; Cayman Chemical, Ann Arbor, MI) at concentrations of 1–250 μM at 37°C for 16 h unless otherwise indicated. To pharmacologically inhibit RhoA, cells were pretreated with C3 toxin from Clostridium botulinum (List Biological, Campbell, CA), 1 μg/ml by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as a carrier molecule 24 h prior to cell harvest, as described (55), which results in a transfection rate of >90% of cells as demonstrated by the inhibition of stress fibers (10). Rabbit polyclonal antibodies to FAK and pFAK were from Biosource (Camarillo, CA). Anti-paxillin was from Upstate (Waltham, MA). Lysophosphatidic acid (LPA; 100 μM) was from Calbiochem (San Diego, CA). Other reagents were obtained from Sigma unless specified. Cell viability was measured by using Trypan blue exclusion. Monoclonal antibodies specific for RhoA were obtained from Cytoskeleton (Denver, CO; catalog no. ARH01-A).

To measure RhoA activation, we used the Rhotekin binding domain affinity precipitation assay for RhoA-GTP according to the manufacturer’s protocol (Cytoskeleton), as described (54). SDS-PAGE was performed as described (22). Bands were detected with enhanced chemiluminescence (ECL-Super Signal; Pierce, Rockford, IL), and images on radiographic film were quantified by using a GS700 Bio-Rad densitometer and QuantityOne analysis software (Bio-Rad, Hercules, CA).

The rate of proliferation of IEC-6 cells was measured by using a colorimetric 3-(4,5-dimethylthiazol-2-yI)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay (Promega, Madison, WI) in cells that were plated on glass coverslips, serum-starved overnight, then treated with detaNONOate (250 μM, 20 h) with 5 mg/ml MTT and then by measuring absorbance at 550 nm, according to the manufacturer’s instructions (41).

For immunohistochemical studies, cells or tissues were processed as described (21) and fluorescent images were captured by using an Olympus Fluoview 1000 confocal microscope under a ×60 oil-immersion objective and standard filter sets. Quantification of the fluorescent signal in immunostained tissues was performed by using Metamorph software (Universal Imaging, Downingtown, PA). Digital images were prepared and labeled by using Adobe Photoshop 7.0 software.

To measure the rate of apoptosis, IEC-6 cells were serum-starved, treated with detaNONOate (250 μM, 20 h), fixed in 4% paraformaldehyde, and immunostained with affinity-purified antibodies against cleaved caspase-3 (Cell Signaling Technology, Danvers, MA). Transfected cells were identified by using anti-hemagglutininA antibodies (Chemicon International, Temecula, CA), followed by Cy3-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA).

Induction of experimental NEC. The following experimental protocol was approved by the Animal Research and Care Committee of the Children’s Hospital of Pittsburgh (protocol no. 0805). NEC was induced in newborn Sprague-Dawley rat pups or mice that were either wild-type C57Bl/6 or deficient in the enzyme iNOS (NOS2, strain B6.129P2-Nos2tm1Laulf, stock no. 002609; Jackson Laboratory, Bar Harbor, ME) by the administration of enteral formula and the induction of hypoxia (5% oxygen for 2 min prior to each feeding) twice daily for 4 days (43). We and others (12, 17, 43, 51) have demonstrated that this experimental protocol induces an intestinal inflammation in animals that resembles clinical NEC. Control animals remained with their mothers and received breast milk. To measure enterocyte migration, animals were injected intraperitoneally with bromodeoxyuridine (50 mg/kg 5’-BrdU; Sigma, St. Louis, MO) then were killed 18 h later. Samples of terminal ileum were then immunostained by using anti-BrdU antibodies as described (27). Enterocyte migration was expressed by measuring the distance from the bottom of the crypt to the foremost labeled enterocyte. When indicated, serum was obtained by cardiac puncture immediately after death, and the concentration of IL-6 (R&D Systems, Minneapolis, MN) was determined by ELISA according to the manufacturer’s instructions. The severity of experimental NEC was graded on a scale of 0 (normal) to 4 (severe intestinal inflammation and necrosis) by two blinded observers according to the description by Nadler and colleagues (43).

Plasmid construction and site-directed mutagenesis. Construction of plasmids that express wild-type SHP-2 or dominant negative SHP-2 (R-E mutant, R465E-SHP2) C459S-SHP-2 was described and characterized previously (1). IEC-6 cells were stably transfected with wild-type or dominant negative SHP-2 by using the retroviral vector REBNA/IRES/GFP (described in Refs. 1 and 66). Infected cells were screened for increased expression of SHP-2 by SDS-PAGE. Cells that were found to have levels increased three- to fourfold over endogenous expression were further analyzed as described below.

Wild-type and/or dominant negative hemagglutininA-tagged constructs of RhoA, RhoB, and RhoC were obtained from the UMR cDNA Resource Center (University of Missouri-Rolla, Rolla, MO) and were transfected into IEC-6 cells by using Lipofectamine 2000 (Invitrogen) as a carrier, according to the manufacturer’s instructions.

Determination of enterocyte migration and intestinal restitution. IEC-6 cells were grown on glass coverslips to 100% confluence and were then serum-starved for 12 h, scraped with a cell scraper, and transferred to the stage of an Olympus IX71 inverted microscope (Olympus, Melville, NY) and perfused with DMEM plus 10 mM HEPES (pH 7.4) at 0.5 ml/h. Images were taken every 5 min for 20 h and were analyzed by using Metamorph software. A calibration scale was obtained, and the migration rate was determined by measuring the mean distance traveled by 15 cells per field over the course of the experiment. Measurements were obtained from cells that were selected both at the leading edge and several rows back.

Assessment of cell-matrix tension. Measurements of cell-matrix tension were performed based on the methodology of Grinnell et al. (20). IEC-6 cells (2 × 105) were plated on top of collagen matrices that were formed in six-well plates [~98% bovine collagen type I (Cohesion Technologies, Palo Alto, CA), 0.1 M NaOH, and 10× PBS mixed together in an 8:1:1 ratio]. After 24 h at 37°C, collagen discs were gently lifted from the wells by using a scraper (t = 0 h) and were treated with fresh media and either LPA (100 μM) or detaNONOate for 24 h to allow contraction to occur (t = 24 h). Contraction was determined by measurement of the change in diameter of the gel over the 24-h period by two independent observers using a ruler. No change in diameter was observed in the absence of enterocytes, and equal numbers of IEC-6 cells were used in each group (2 × 105).

Measurement of cell attachment. Cell attachment was assessed according to the methodology of Johnson and colleagues (60). In brief, IEC-6 cells were suspended at a density of 2.5 × 105 cells/ml.
after treatment with 0.05% trypsin plus 0.53 mM EDTA in Hanks’ balanced salt solution. Dishes (35 mm) were then coated with Matrigel (40 µl/ml; BD Biosciences, Billerica, MA) and were incubated with Dulbecco’s phosphate-buffered saline (DPBS)/0.1% BSA for 1 h at room temperature to block nonspecific binding sites. Cells were then plated at 3 × 10⁴ cells/cm² and were incubated for 24 h in the presence or absence of detaNONOate (250 µM), then were placed on ice and washed three times with ice-cold DPBS, fixed with 10% formaldehyde-2% Triton X-100 in DPBS for 10 min at room temperature, and washed three times with DPBS and once with water. The attached cells were immediately photographed such that three pictures were taken from each dish at ×100 magnification, and the total number of cells per area was counted using Metamorph software.

Statistics. Data presented are means ± SE. Comparisons are by two-tailed Student’s t-test, with statistical significance accepted for P < 0.05.

RESULTS

Experimental NEC is characterized by mucosal NO release and impaired intestinal restitution. We first examined the release of NO and the extent of intestinal restitution in an animal model of NEC. As is shown in Fig. 1, A and B, the induction of NEC was characterized by blunting and loss of villi, edema of the lamina propria, and the influx of inflammato-

Fig. 1. Nitric oxide (NO) is released in the mucosa of animals with experimental necrotizing enterocolitis (NEC). Intestinal injury was induced in newborn rats and mice by using a combination of gavaged formula and systemic hypoxia. A: normal villus architecture of the ileum in control, breast-fed rats. B: histopathology of rats with experimental injury resembling NEC showing inflammation of the lamina propria, edema, and necrosis similar to that observed in human NEC. C–F: 3-nitrotyrosine (3-NT) staining of the terminal ileum obtained from control rats (C) and mice (E) and those with experimental NEC (D and F, respectively) showing increased expression in the mucosa of rats with NEC (DRAQ5 nuclear staining in blue, 3-NT staining in green). G: means ± SE of 3-NT expression in control and NEC rats as quantified in MATERIALS AND METHODS. H–I: to assess enterocyte migration along the crypt-villus axis, control and NEC mice were injected with bromodeoxyuridine (BrdU) 18 h prior to death and then immunostained for BrdU (arrows indicate position of peroxidase staining). H: in control mice, most BrdU staining is accumulated at varying positions along the villi (left, arrows). In NEC animals, the majority of the BrdU uptake remains in the crypts (right, arrows), demonstrating a significant impairment in migration. I: rate of migration and the percentage of maximal crypt height achieved by migrating enterocytes is significantly decreased in NEC animals compared with controls. Data are means ± SE of 3 experiments. A minimum of 100 cells were counted per experiment. Bar = 50 µm.
tory cells. To assess a role for NO in the pathogenesis of NEC, we measured the mucosal release of NO by immunostaining the terminal ilea of rats (Fig. 1, C and D) and mice (Fig. 1, E and F) with and without NEC with antibodies against 3-nitrotyrosine (3-NT), a reactive product of NO reflective of local NO release (63). As can be seen, 3-NT immunoreactivity was significantly increased in the intestinal mucosa of animals with experimental NEC (Fig. 1, D and F) compared with untreated animals (Fig. 1, C and E, and quantification in Fig. 1 G). To measure intestinal restitution, samples of terminal ileum that had been obtained from control mice and those with experimental NEC were immunostained with anti-BrdU antibodies. Consistent with our previous results (10), intestinal restitution was significantly decreased in NEC compared with breast-fed controls (Fig. 1 H), reflected in a decrease in the rate of migration of enterocytes along the crypt-villus axis and a decrease in the maximal height attained, as quantified in Fig. 1 I.

The above findings raise the possibility that NO release could potentially impair enterocyte migration. To assess this directly, IEC-6 enterocytes were allowed to migrate across a scraped wound in the presence or absence of NO. As is shown in Fig. 2, A, B, and E, the migration of control cells resulted in closure of the wounded area by 20 h. By contrast, treatment with NO led to a dose-dependent decrease in the rate of enterocyte migration (Fig. 2, C, D, and E). To confirm that IEC-6 cells remain viable after treatment with detaNONOate, cells were exposed to the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (100 μM) 12 h after NO treatment and were allowed to migrate for an additional 14 h. This led to a partial restoration of the rate of enterocyte migration (Fig. 2E). As is shown in Fig. 2, F and G, rates of proliferation and apoptosis were decreased in the presence of detaNONOate, two additional factors that may also affect rates of restitution. Taken alongside the results of Fig. 1, these results demonstrate that NEC is associated with the local accumulation of NO in the intestinal mucosa and impaired intestinal restitution and that NO significantly impairs the rate of enterocyte migration.

**NO increases the activity of Rho-GTPase in enterocytes.** Migration of many cell types is tightly regulated by dynamic modulation of the cytoskeleton under the control of the small-molecular-weight GTPase RhoA (28). We therefore next sought to determine whether the inhibitory effects of NO on enterocyte migration were related to changes in RhoA activity. As is shown in Fig. 3A, NO increased the activity of RhoA-GTPase in enterocytes in a dose-dependent manner, which was associated with a corresponding dose-dependent redistribution of F-actin into fiberlike bundles, consistent with the known effects of active RhoA in forming stress fibers (28) (Fig. 3B, C, D, and E).

### Figure 2. NO impairs the migration of enterocytes. IEC-6 cells were plated to confluence in the absence (A, B) or presence (C, D) of diethylaminetriamine-NONOate (detaNONOate; 250 μM), scraped, and allowed to migrate over 20 h. The position of the leading edge of the cells at the beginning and end of the experiment is shown (dashed line). Bar = 20 μm. E: effect of varying doses of detaNONOate or addition of the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) on the rate of enterocyte migration. Data are representative of at least 10 separate experiments. *P < 0.05 vs. control by Student’s t-test. F: IEC-6 cells were serum-starved overnight and then treated with detaNONOate (250 μM) for 20 h and assessed for proliferation rate in a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. *P < 0.05 for absorption at 550 nm compared with untreated cells, representative of 3 separate experiments. G: IEC-6 cells were plated overnight on glass coverslips, serum-starved, then treated with detaNONOate (250 μM, 20 h) and immunostained with antibodies against cleaved caspase-3, a marker of apoptosis. *P < 0.05 vs. untreated (Ctrl) cells, representative of 3 separate experiments.
After treatment with increasing concentrations of detaNONOate as described in MATERIALS AND METHODS. Bar = 10 μm. C: HeLa cells (positive control) and IEC-6 cells that had been treated with 100 μM of detaNONOate or left untreated were subjected to SDS-PAGE and were immunoblotted with anti-RhoA antibodies.

20 – i. iv. Of note, NO treatment did not affect the expression of Rho-modifying factors that have been shown to regulate RhoA activity in other systems (61), including Vav3, a known GTPase activating protein (Fig. 3C) (3). These findings suggest the possibility that the changes in the level of RhoA activity occur through an alternate pathway, as will be assessed in further detail below.

To confirm that the effects of NO on stress fiber formation in IEC-6 cells acted via RhoA as opposed to other commonly expressed Rho isoforms, a genetic approach was undertaken. As shown in Fig. 4, compared with the relative paucity of stress fibers that were observed in untreated IEC-6 cells that had been transfected with hemagglutinin-tagged wild-type RhoA (Fig. 4, A and B), NO treatment caused a marked increase in the formation of stress fibers in IEC-6 cells (Fig. 4, C and D). By contrast, transfection of IEC-6 cells with hemagglutinin-tagged dominant negative RhoA prevented the induction of stress fibers in response to NO (Fig. 4, E and F), whereas transfection with dominant negative RhoB and RhoC, two isoforms of RhoA (69), did not prevent the formation of stress fibers in response to NO treatment. In addition to the evidence displayed in Fig. 3, these findings provide evidence that NO induces the formation of stress fibers in IEC-6 enterocytes in a RhoA-dependent manner.

NO leads to a Rho-GTPase-dependent increase in the expression of pFAK in enterocytes and a corresponding increase in focal adhesion formation. Given that NO was found to inhibit enterocyte migration and to activate RhoA (Figs. 2 and 3, respectively), we next sought to determine the mechanisms involved. The migration of many cell types is tightly regulated by the formation and dissolution of highly organized protein structures that anchor the plasma membrane to the underlying matrix, called focal adhesions (see Ref. 57 for a recent review). The regulation of focal adhesion formation is governed in part by the activity of the enzyme FAK (29), whose activation is partially dependent on RhoA activity (6, 36). Having shown that NO increases RhoA activity in enterocytes, we next considered whether NO affected the phosphorylation of FAK. As is shown in Fig. 5A, NO caused a dose-dependent increase in the phosphorylation of FAK in IEC-6 cells. Treatment of enterocytes with the Rho inhibitor C3 exotoxin prevented the increase in the phosphorylation of FAK by NO, confirming the dependence on Rho in the pathways leading to FAK phosphorylation (Fig. 5B). Given the importance of pFAK in the formation of focal adhesions (57), we next tested whether the increase in pFAK expression in response to NO could increase the formation of focal adhesions in enterocytes. As is shown in Fig. 5C, NO treatment of IEC-6 cells caused a marked increase in pFAK immunostaining compared with untreated cells (Fig. 5C, i and iv). In parallel, NO caused an increase in the immunoreactivity of the focal adhesion marker paxillin, indicating that focal adhesion formation was increased after NO treatment. (Fig. 5C, ii and v). NO was also observed to cause an increase in the expression of paxillin and phosphopaxillin, as measured by SDS-PAGE (Fig. 5D).

The persistent formation of focal adhesions in enterocytes by NO may be expected to lead to an increase in the strength with which enterocytes are bound to the underlying matrix, potentially impairing their migration (26, 47). We therefore next hypothesized that the adhesiveness of enterocytes to the underlying matrix would be increased by NO. This hypothesis was tested directly by assessing the ability of IEC-6 cells to contract a collagen matrix in the presence or absence of NO, as we have described previously (8, 10). As shown in Fig. 5E, NO treatment significantly increased enterocyte-matrix contraction of IEC-6 cells compared with untreated cells. Treatment of cells with LPA, which is known to increase focal adhesions through an increase in Rho-GTPase (48), led to a significant increase in cell-matrix contractility compared with untreated cells and served as a positive control in providing evidence that RhoA activity leads to increased enterocyte contraction. These findings were confirmed in an adhesion assay, in which NO was found to significantly increase the adhesion of IEC-6 cells

AJP-Gastrointest Liver Physiol • VOL 292 • MAY 2007 • www.ajpgi.org

Downloaded from http://ajpgi.physiology.org/ by 10.220.33.5 on July 6, 2017
to a Matrigel matrix (Fig. 5F). Together, these findings provide a functional correlate of the increased formation of focal adhesions induced by NO, as observed in Fig. 5C.

The severity of intestinal inflammation and expression of pFAK are increased in the terminal ilea of newborn wild-type vs. iNOS$^{-/-}$ mice induced to develop NEC. The above data indicate that NO exposure leads to both an increase in the expression of pFAK in IEC-6 cells and an impairment in enterocyte migration in vitro. To assess the potential in vivo significance of these findings, we next sought to assess both the severity of disease and the expression of pFAK in the terminal ileal mucosa of newborn wild-type and iNOS$^{-/-}$ mice after the induction of experimental NEC. The exposure of wild-type newborn mice to enteric formula and hypoxia led to the observation of patchy necrosis of the small intestine resembling NEC (Fig. 6, A and B). To evaluate the severity of NEC that developed, sera were collected and assayed for the release of the proinflammatory cytokine IL-6, an established marker of NEC severity (44, 45, 56). As shown in Fig. 6C, the release of IL-6 was significantly greater in wild-type mice after exposure to formula and hypoxia compared with breast-fed controls yet was markedly reduced in iNOS$^{-/-}$ mice under the same experimental conditions. The reduction in IL-6 release that was observed in iNOS$^{-/-}$ mice after induction of experimental NEC correlated with a decrease in the extent of mucosal inflammation and necrosis compared with wild-type littermates (Fig. 6, D–F). To assess pFAK expression, mucosal scrapings were obtained from wild-type and iNOS$^{-/-}$ mice after exposure to formula and hypoxia (Fig. 6G). In wild-type mice, the expression of pFAK was significantly increased in the terminal mucosa of animals that were induced to develop NEC compared with breast-fed controls. By contrast, iNOS$^{-/-}$ mice failed to demonstrate a significant increase in the expression of pFAK in the intestinal mucosa after exposure to similar NEC-inducing conditions (Fig. 6G). These findings together raise the possibility that NO release during the development of NEC leads to an increase in pFAK expression in enterocytes in vivo, in confirmation of the in vitro findings described in Fig. 5.

NO activates RhoA in enterocytes in a SHP-2-dependent manner. Having shown that NO leads to an increase in pFAK expression in enterocytes in vitro and in vivo and to a RhoA-dependent increase in focal adhesiveness in vitro, we next sought to determine the potential mechanisms involved. To do so, we focused on the activity of the tyrosine phosphatase SHP-2, which has been shown to modulate the activity of RhoA in various migrating cells (39). NO treatment resulted in a dose-dependent increase in the expression of SHP-2 in IEC-6 cells transiently transfected with cDNA encoding hemagglutinin-tagged constructs encoding either wild-type (WT-) RhoA, dominant negative (DN-) RhoA, DN-RhoB, or DN-RhoC. Cells were then either left untreated (A and B) or were treated with detaNONOate (250 μM) for 14 h (C–J), then were immunostained with antibodies against hemagglutinin and rhodamine phalloidin and were examined by confocal microscopy such that the distribution of F-actin appears in red (phalloidin; A, C, E, G, and I) and the hemagglutinin stain identifying transfected cells appears in green (anti-HA; B, D, F, H, and J). Arrows identify the formation of stress fibers in NO-treated cells transfected with WT-RhoA, DN-RhoB, and DN-RhoC and indicate the lack of stress fibers in untreated cells and those transfected with DN-RhoA. There is no effect of inhibition of RhoB or RhoC on NO-induced stress fiber formation. Bar = 10 μM. Representative images are from 3 individual experiments with over 50 transfected cells per experiment.
enterocytes, suggestive of an increase in SHP-2 activation in response to NO (Fig. 7, A and B). To determine a possible role for SHP-2 in the activation of RhoA by NO in enterocytes, stable IEC-6 lines expressing a dominant negative SHP-2 were generated (see MATERIALS AND METHODS). As is shown in Fig. 7, C and D, IEC-6 cells expressing wild-type SHP-2 demonstrated an increase in activated RhoA after NO treatment, similar to that observed in nontransfected cells. By contrast, IEC-6 cells expressing dominant negative SHP-2 showed no change in RhoA activation after NO treatment. The morphology of the actin cytoskeleton of wild-type SHP2-IEC6 cells was similar to that of nontransfected controls (Fig. 7D, i and iii). Although actin stress fiber formation was found to be increased in wild-type SHP2-IEC6 cells after NO treatment (Fig. 7D, ii),
NO treatment of dominant negative SHP2-IEC6 cells did not cause an increase in stress fiber morphology (Fig. 7D, iv). Together, these studies support the conclusion that SHP-2 activity is required for the activation of RhoA and stress fiber formation by NO.

**Dominant negative SHP-2 prevents NO-dependent phosphorylation of FAK and reverses the inhibitory effects of NO on enterocyte migration.** To investigate whether SHP-2 activation is required for the NO-induced phosphorylation of FAK and increase in focal adhesions, both wild-type and dominant negative SHP2-IEC6 cells were treated with NO and the expression of phosphorylated and nonphosphorylated FAK was assessed. As shown in Fig. 8A, wild-type SHP-2-transfected cells demonstrated a significant increase in pFAK expression after NO treatment by SDS-PAGE, which was not observed in IEC-6 cells that stably expressed dominant negative SHP-2. Immunofluorescent studies revealed that NO treatment caused an increase in the expression of pFAK in IEC-6 cells expressing wild-type SHP-2 (Fig. 8B, ii vs. i), which was not observed in IEC-6 cells that were transfected with dominant negative SHP-2. Importantly, although NO treatment led to an increase in cell-matrix adhesiveness in cells transfected with wild-type SHP-2 (Fig. 8C), cells transfected with dominant negative SHP-2 did not demonstrate a significant change in cell-matrix adhesiveness after NO treatment. Together, these findings support the conclusion that NO increases focal adhesion formation in a SHP-2-dependent manner.

Given the fact that enhanced cell-matrix adhesiveness leads to impaired migration (26, 47), we finally considered whether SHP-2 could mediate the inhibitory effects of NO on enterocyte migration. Without NO treatment, the rate of migration of dominant negative SHP-2-transfected cells was similar to that of wild-type SHP-2-transfected controls (Fig. 8D). Treatment of wild-type SHP-2-transfected IEC-6 cells with NO caused a significant reduction in the rate of enterocyte migration compared with untreated cells, similar to that seen in nontransfected controls (Fig. 8D). Strikingly, NO did not inhibit the migration of IEC-6 cells expressing dominant negative SHP-2 (Fig. 8D). Together, these results indicate a critical role for SHP-2 in the inhibitory effects of NO on focal adhesion formation and migration of enterocytes.

**DISCUSSION**

In the current study, we report the novel findings that NO causes a dose-dependent inhibition in the rate of enterocyte migration and that this effect occurs through a SHP-2-dependent activation of RhoA and an increase in focal adhesions via phosphorylation of FAK. Given the observation that pFAK expression is increased in the mucosa of wild-type but not iNOS−/− mice subjected to enteric formula and hypoxia, these studies seek to shed light on the mechanisms by which NO contributes to the pathogenesis of intestinal inflammation in diseases like NEC. Previous studies have revealed that the effects of NO on the intestine are biphasic in nature and are dose-dependent (7). At relatively low doses, NO exerts a protective action against mucosal injuries, in part through potential vasodilator effects resulting in enhanced mesenteric blood flow (13, 35, 65). However, high levels of NO produced by iNOS may play a potent role as a cytotoxic agent (24, 49), leading to increased permeability of the intestinal epithelium (67). The inhibition in enterocyte migration by NO would thus be expected to compound the overall effects of NO on barrier injury by causing a “double hit” to the intestine, first by injuring the mucosa, then by preventing its restitution. An understanding of the mechanisms by which NO acts to inhibit enterocyte migration is therefore of great interest, to develop strategies to potentially restore restitution and improve therapy for diseases like NEC.
Although we now demonstrate that NO inhibits migration of enterocytes, the effects of NO on migration of other cells appear to be cell type- and stimulus-dependent. Specifically, previous authors have shown that NO has a stimulatory effect on the migration of endothelial cells (38) and primary aortic smooth muscle cells (4). In contrast, NO inhibits the migration of gastric epithelial cells (31), hepatic stellate fibroblasts (37), neutrophils (46), endothelial cells (33), and aortic smooth muscle cells (25). The divergent effects of NO on cell migration may in part reflect cell-specific or dose-dependent differences between experiments. It is noteworthy that the concentrations of detaNONOate used in the current study were within the range that may be observed in vivo in diseases of intestinal inflammation, including NEC (5, 16, 34, 72), when the long half-life of this particular NO donor is taken into account (30). However, it is likely that the interaction of NO with a variety of pro- or antimigratory signaling pathways determines whether the cell assumes a motile or stationary phenotypic (14). The identification of putative NO-specific targets and their regulation in migrating cells is critical to fully explain the role of NO in regulating cell migration in vivo and in vitro.

A major finding of the current study is that NO causes a dose-dependent increase in the activity of RhoA, with a corresponding rearrangement in the actin cytoskeleton (Fig. 2). Previous studies have examined an association between RhoA and NO release. In epithelial, vascular smooth muscle, and transformed brain cells, Rho activity modulates the expression of iNOS (42, 52, 70), leading to alterations in NO levels, and Chang et al. (11) have shown that NO decreases RhoA activity in primary aortic smooth muscle cells. Based on our current observations, we now propose that NO-mediated activation of RhoA plays a critical role in mediating the inhibition of migration of enterocytes by NO. Given the multiple signaling pathways likely to be affected by NO and RhoA, NO could conceivably act directly on RhoA or act through intermediary proteins. It is noteworthy that the expression of the activating...
proteins Vav3 and p190RhoGAP do not appear to be influenced by NO (Fig. 3C), suggesting that parallel NO-mediated signaling pathways exist. One such pathway involves the SH2-containing protein tyrosine phosphatase SHP-2, which has been shown to regulate RhoA activity in embryonic fibroblasts (62) and to be necessary and sufficient for the Rho-dependent motility of aortic smooth muscle cells (11). We now demonstrate that inhibition of SHP-2 in IEC-6 cells leads to an inhibition of NO-mediated activation of RhoA and a reversal of the inhibitory effects of NO on enterocyte migration. Based on these findings, we now propose that after exposure to NO, SHP-2 dephosphorylates and inactivates a Rho-GTPase-associated protein in enterocytes, leading to the activation of RhoA, phosphorylation of FAK, increased cell attachment, and impaired migration.

In previous studies from our laboratory (10, 51), we have demonstrated that high levels of endotoxins, which may be released from gram-negative bacteria during the process of translocation across the inflamed intestinal epithelium, inhibit enterocyte migration through an increase in the formation of focal adhesions and a corresponding increase in cell-matrix adhesiveness. The current study therefore provides evidence whereby the activity of a key inflammatory mediator, NO, results in similar effects to those observed after exposure to high levels of endotoxin. The impairment in intestinal restitution induced by NO (Fig. 2) may be expected to exacerbate the
degree of intestinal injury by enabling ongoing bacterial translocation across the disrupted mucosal barrier (15, 18, 50). This may account in part for the reduction in severity of NEC observed in iNOS−/− mice compared with wild-type littermates, in addition to the cytopathic effects that NO may be having on the intestinal barrier. Together, these studies may help to partially explain the pathways that underlie the finding of persistent intestinal failure that characterizes intestinal inflammatory disorders, including NEC.

In conclusion, we have now shown that the release of NO as occurs during neonatal NEC leads to a dose-dependent inhibition in enterocyte migration. This effect appears to be mediated via a SHP-2-dependent activation of RhoA and an increase in focal adhesions, leading to enhanced cell-matrix adhesiveness. We propose that an understanding of the mechanisms by which cytokines such as NO modulate enterocyte matrix may provide novel therapeutic insights into the regulation of enterocyte migration during diseases of intestinal inflammation such as NEC.

ACKNOWLEDGMENTS

We thank Patricia Boyle and Catherina Wong for assistance with experiments, and Timothy Billiar for assistance with experimental design.

Present address of H. Ford and J. Upperman: Division of Pediatric Surgery, Childrens Hospital Los Angeles, and Department of Surgery, University of Southern California.

GRANTS

This work was supported by National Institutes of Health Grants R01-GM-78238 (D. J. Hackam), R01-AI-49473 (H. R. Ford), and R01-AI4032 (H. R. Ford), and the State of Pennsylvania Tobacco Settlement Fund. C. L. Leaphart is supported by a Loan Repayment Grant from the National Institutes of Health.

REFERENCES


NITRIC OXIDE IMPAIRS ENTEROCYTE MIGRATION VIA RhoA

G1357
Nitric oxide impairs enterocyte migration via RhoA