Peroxynitrite inhibits enterocyte proliferation and modulates Src kinase activity in vitro

Douglas A. Potoka, Jeffrey S. Upperman, Xiao-Ru Zhang, Joshua R. Kaplan, Seth J. Corey, Anatoly Grishin, Ruben Zamora, and Henri R. Ford. Peroxynitrite inhibits enterocyte proliferation and modulates Src kinase activity in vitro. Am J Physiol Gastrointest Liver Physiol 285: G861–G869, 2003. First published July 3, 2003; 10.1152/ajpgi.00412.2002.—Overproduction of nitric oxide (NO) or its toxic metabolite, peroxynitrite (ONOO−), after endotoxemia promotes gut barrier failure, in part, by inducing enterocyte apoptosis. We hypothesized that ONOO− may also inhibit enterocyte proliferation by disrupting the Src tyrosine kinase signaling pathway, thereby blunting repair of the damaged mucosa. We examined the effect of ONOO− on enterocyte proliferation and Src kinase activity. Sprague-Dawley rats were challenged with LPS or saline, whereas intestinal epithelial cell line cells were treated with ONOO− or decomposed ONOO− in vitro. Enterocyte proliferation in vivo and in vitro was measured by 5-bromo-2′-deoxyuridine (BrdU) or [3H]thymidine incorporation. Src kinase activity in vivo and ONOO− treatment blunted the activity of Src and its downstream target, focal adhesion kinase, in a time-dependent manner. ONOO− blocked mitogen (FBS, EGF)-induced enterocyte proliferation and Src phosphorylation while increasing Src nitration. Thus ONOO− may promote gut barrier failure not only by inducing enterocyte apoptosis but also by disrupting signaling pathways involved in enterocyte proliferation.


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SUSTAINED OVERPRODUCTION OF nitric oxide (NO) in the gastrointestinal tract has been demonstrated in a variety of inflammatory conditions in vivo (4, 9, 14, 26, 27, 29, 39, 40). Evidence suggests that such excess nitric oxide (NO) production may promote cellular injury and disrupt the intestinal epithelial barrier. Previously, we have shown that the inducible isoform of NO synthase (iNOS) is upregulated in the intestinal epithelium of newborn infants with necrotizing enterocolitis (NEC) (14). The iNOS protein colocalizes with enterocyte apoptosis at the villus tips and with immuno-reactivity to 3-nitrotyrosine, a molecular marker of reactive nitrogen intermediates such as peroxynitrite (ONOO−) in the intestinal epithelium and lamina propria. Furthermore, iNOS upregulation, nitrotyrosine immunoreactivity and enterocyte apoptosis have also been demonstrated in conditions such as endotoxemia, inflammatory bowel disease (IBD), and Helicobacter pylori gastritis, as well as in animal models of colitis and ileitis (4, 9, 12, 26, 27, 29, 39–41). These findings suggest that ONOO−, a toxic metabolite formed by the reaction of NO with superoxide, may be a key mediator of mucosal injury and gut barrier failure in vivo.

One mechanism by which ONOO− may promote mucosal injury is by inducing enterocyte apoptosis. Indeed, intestinal epithelial cell apoptosis has been shown to occur in a variety of conditions associated with gastrointestinal inflammation including endotoxemia, NEC, IBD, and Helicobacter pylori gastritis (4, 14, 26, 27, 40). The administration of iNOS inhibitors or NO scavengers decreased enterocyte apoptosis and immunoreactivity to 3-nitrotyrosine and ameliorated gut barrier failure after endotoxemia (12, 41). Moreover, we and others have demonstrated that ONOO− can induce enterocyte apoptosis in vitro (33, 37, 44). However, a central feature of the intestinal epithelial barrier is its ability to repair itself after mucosal injury. The integrity of the intestinal mucosa depends on the continued proliferation, migration, and differentiation of the crypt enterocytes (48). Interference with these processes may promote or exacerbate mucosal injury. For example, decreased proliferation of crypt enterocytes has been shown to be part of the characteristic morphological changes seen in the intestine after burn injury (46). We propose that in conditions associated with overproduction of NO or ONOO−, gut barrier failure may result from an imbalance between accelerated epithelial injury and blunted tissue repair mechanisms. The effect of ONOO− on the proliferation side of this equation has not been previously explored.

Regulation of intestinal epithelial proliferation and differentiation is not completely understood (31). The
Src family of nonreceptor tyrosine kinases may play a key role in enterocyte proliferation. For example, higher levels of Src protein are detected in proliferating crypt enterocytes than in villus enterocytes, and Src activity is elevated in colon cancer cells and in areas of epithelial dysplasia in ulcerative colitis (6–8, 42). Activation of Src kinase results in phosphorylation of downstream targets such as Shc, phosphatidylinositol-3-kinase, and focal adhesion kinase (FAK), which then diverge along distinct signaling pathways that lead to cellular proliferation, migration, or differentiation (10, 17, 38). Regulation of Src function is dependent on Src phosphorylation at key tyrosine residues. Thus the Src signaling pathway may be a potential site for modulation by ONOO−, because ONOO− is known to nitrate tyrosine residues (3).

We hypothesized that ONOO− may inhibit enterocyte proliferation by disrupting Src kinase-dependent signaling pathways. In this study, we examined the effect of LPS on enterocyte proliferation in a rat model of endotoxemia in vivo and the effect of ONOO− on enterocyte proliferation and Src kinase activity in vitro by using the IEC-6 rat intestinal epithelial cell line.

MATERIALS AND METHODS

Reagents. ONOO− and decomposed ONOO− were obtained from Alexis Biochemicals (San Diego, CA). Recombinant human EGF was obtained from R&D Systems (Minneapolis, MN). 5-Bromo-2′-deoxyuridine (BrdU) and the superoxide generator, pyrogallol, were obtained from Sigma (St. Louis, MO). The NO donor, N-nitroso-N-acetyl-penicillamine (SNAP), was synthesized from NaNO2 and N-acetyl-D,L-penicillamine (Sigma, St. Louis, MO) as previously described (15). Polyclonal Src antibody (sc-18), rabbit polyclonal FAK antibody, and monoclonal phosphotyrosine antibody (sc-7020) were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Rabbit polyclonal phosho-FAK (Tyr576/577) antibody was obtained from Cell Signaling Technology (Beverly, MA). Polyclonal nitrotyrosine antibody (06–284) was obtained from Upstate Biotechnology (Lake Placid, NY). The pMv-Src vector carrying the chicken v-Src gene under the control of the cytomegalovirus (CMV) promoter was a generous gift of Dr. Ed Prochownik, Children’s Hospital of Pittsburgh (Pittsburgh, PA). The pcDNA3.1 control vector was purchased from Invitrogen (Carlsbad, CA).

Measurement of enterocyte proliferation in vivo. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing between 250 and 300 g were acclimatized for a minimum of 1 wk before experimentation. The experimental protocol was approved by the Animal Research and Care Committee of the Children’s Hospital of Pittsburgh. The animals were challenged with 10 mg/kg of LPS (Escherichia coli 0127:BS; Sigma) intraperitoneally or with equal volume of saline (12). After 23 h, the rats received 50 mg/kg BrdU ip and were then killed 1 h later. Segments of terminal ileum and colon were harvested, fixed in formalin, immunostained with anti-BrdU antibody (Amersham, Arlington Heights, IL), and then counterstained with hematoxylin. Enterocyte proliferation was determined by counting the number of BrdU-positive cells per crypt (15 crypts) by using light microscopy (Olympus BH-2 microscope).

Cell culture. The rat IEC-6 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in tissue culture medium consisting of Dulbecco’s modified Eagle’s medium with 4.5 g/ml glucose (Bio-Whittaker, Walkersville, MD) supplemented with 5% FBS (Bio-Whittaker), 0.02 mM glutamine (GIBCO, Grand Island, NY), 0.1 U/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO) at 37°C and 10% CO2. Cells from passages 3 through 26 were used for experiments.

ONOO− treatment. ONOO− stock solution was stored at −80°C. The concentration of ONOO− was monitored spectrophotometrically before each experiment by measuring absorbance at 302 nm (ε284 nm = 1,670 M cm). Solutions of 10 mM ONOO− in 0.3 N NaOH were used for experiments. For all experiments, decomposed ONOO− was used for the negative control and made in similar concentrations in 0.3 N NaOH as for ONOO−. IEC-6 cells were washed twice with PBS (GIBCO) before ONOO− treatment. ONOO− or decomposed ONOO− was then added to the side of the well and then mixed into the solution by swirling for 20 s.

In vitro proliferation assay. Approximately 75% confluent IEC-6 monolayers grown in 96-well plates were cultured in serum-free medium for 48 h. After the monolayers were washed with PBS, the cells were then treated with 50 μM ONOO− or with 50 μM decomposed ONOO− for 5 or 10 min. After treatment, the cell monolayers were then washed twice with PBS and then treated with 1 ml of regular tissue culture medium. Cells were harvested and [3H]thymidine incorporation was measured by liquid scintillation spectrometry.

To determine the effect of ONOO− on PBS- or EGF-induced proliferation, 75% confluent IEC-6 monolayers in two-chamber slides were grown in serum-free medium at 37°C and 10% CO2 for 48 h. The monolayers were washed twice with PBS and then treated with 50 μM ONOO− or with 50 μM decomposed ONOO− for 10 min. The monolayers were then washed with 1 ml of PBS and then treated with 1 ml of regular tissue culture medium, 1 ml of serum-free tissue culture medium, or 1 ml of serum-free medium plus 200 ng/ml human recombinant EGF or 5% FBS for 4 h at 37°C and 10% CO2. After this incubation, 10 μM BrdU was added to each well for 1 h at 37°C and 10% CO2. After incubation, the upper chambers were removed from the slides, and BrdU incorporation was analyzed immunohistochemically by using a commercially prepared BrdU staining kit (Zymed, San Francisco, CA). Slides were examined by using a Olympus BH-2 microscope. The degree of proliferation was defined as the proportion of BrdU positive cells per 5–10 high-power fields.

Src kinase activity assay. IEC-6 monolayers grown to ~75% confluence were washed with PBS twice and then treated with buffer or with 50 μM ONOO− or decomposed ONOO− for 1, 5, 15, and 30 min. After each treatment time, cells were isolated from the plate by scraping, washed in PBS, and resuspended in 200 μl of lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 1% Nonidet P-40 (NP-40), 10% glycerol, 10 ng/ml aprotinin, 10 ng/ml leupeptin, 2 mM sodium orthovanadate, and 1 mM PMSF) for 30–45 min at 4°C. The supernatant was collected after centrifugation at 9,500 x g at 4°C for 10 min. The cell lysates were analyzed by using a bicinchoninic acid assay. For each sample (20 μg protein in 200 μl of lysis buffer), 1 μl of Src polyclonal antibody was added for overnight immunoprecipitation of Src at 4°C. To each sample, 15 μl of protein A-Sepharose beads were then added for a 1-h incubation, with constant turning at 4°C. The beads were then pelleted by microcentrifugation for 4°C and washed twice with PBS, 1% NP-40 (1 ml per wash) at 4°C, followed by two washes with buffer (50 mM Tris, 100 mM NaCl, and 1 mM EDTA) at room temperature. After washes, 20 μl of kinase assay buffer (HEPES, pH 7.4, 10 mM MgCl2, 10 mM MnCl2, and 200 μCi/ml [γ-32P]ATP) was...
added to each sample for a 30-min incubation at room temperature. After this incubation, 30 μl of Laemmli sample buffer was added to each sample. Each sample was then boiled for 5 min, and samples were loaded on a 10% polyacrylamide gel and run overnight. Phosphorylated proteins were detected by autoradiography. This assay detects the phosphorylation of immunoprecipitated Src and thus determines the degree of Src activation.

For studies examining the effect of ONOO− on EGF or PBS induction of Src activity, IEC-6 cells were grown to ~75% confluence, as described in In vitro proliferation assay. Cells were first serum starved in serum-free medium at 37°C and 10% CO2 for 48 h. The cells were washed twice with PBS and then treated with 50 μM ONOO− or with 50 μM decomposed ONOO− for 10 min. After treatment, the monolayers were washed with 1 ml of PBS and then replaced with 5 ml of serum-free medium or with 5 ml of serum-free medium plus 200 ng/ml human recombinant EGF or 5% FBS for 15 min at 37°C and 10% CO2. Src kinase assays were then performed as described in Src kinase activity assay.

Transfection experiments. IEC-6 cells grown on 100-mm petri dishes to 75% confluence were transfected with 10 μg plasmid DNA by using the Lipofectamine reagent (Invitrogen) as directed by the manufacturer. IEC-6 cells were transiently transfected with pMV-Src or pcDNA3.1 vector control. After 24 h, cells were collected and seeded into 96-well plates at a density of 10,000 cells/well. After cells attached, they were starved in serum-free medium for 48 h. [3H]thymidine (1 μCi/well) was then added together with ONOO− (50 μM) or equivalent amount of decomposed ONOO−, or Src family inhibitor PP1 (10 μM), or solvent alone. After incubation at 37°C and 5% CO2 for 6 h, cells were harvested and [3H]thymidine incorporation was measured by liquid scintillation spectrometry.

Apoptosis assay. Apoptosis was determined by using flow cytometry with annexin V-FITC and propidium iodide staining as previously described (33, 47).

Western blot analysis. Proteins from cell monolayers were electrophoresed on 8 to 12% SDS-PAGE gels in a Bio-Rad minigel apparatus and blotted onto nitrocellulose membranes as previously described (33). After the membranes were blocked for 1 h with 5% milk in PBS with 0.1% Tween 20, blots were probed for 1 h at room temperature with primary antibody (1:500 dilution). As secondary antibody, horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse IgG was used and detection was performed by enhanced chemiluminescence (Pierce, Rockford IL).

Immunoprecipitation. Treated cells in 100-mm petri dishes or six-well plates were washed twice with ice-cold PBS and lysed in a total of 200 μl of lysis buffer per sample. After incubation at 4°C with turning for 30–45 min, the supernatant was collected after centrifugation at 9,500 g for 20 min. Aliquots of protein (150–200 μg) were prepared in 200 μl lysis buffer. Primary antibody (5 μg) was added to each sample for a 1 h incubation with constant turning at 4°C, followed by the addition of 25 μl of either protein A-Sepharose or protein AG-Sepharose (on the basis of primary antibody used) to each sample for an overnight incubation with constant turning at 4°C. The beads were then pelleted by centrifugation at 350 g for 5 min at 4°C. The beads were washed four times with lysis buffer at 4°C. After the final wash, the beads were resuspended in 30 μl of Laemmli sample buffer, boiled for 3 min, and electrophoresed on 8 to 12% SDS-PAGE gels as described above. Western blot analysis was completed as described previously (33).

Statistical analysis. All data are expressed as means ± SE. For multiple comparisons, ANOVA was used to determine significant differences among groups. Significant differences among individual groups were determined by using Fisher’s exact probability test. P values ≤ 0.05 were considered significant.

RESULTS

Effect of LPS challenge on enterocyte proliferation in vivo. We (12, 41) previously demonstrated that the administration of LPS to Sprague-Dawley rats resulted in intestinal iNOS upregulation, enterocyte apoptosis, and gut barrier dysfunction. The administration of an iNOS inhibitor or an NO scavenger ameliorated the derangement in epithelial barrier function and reduced enterocyte apoptosis and immunoreactivity to 3-nitrotyrosine. To determine whether endotoxemia also impaired enterocyte proliferation, we measured BrdU incorporation in the intestinal epithelium of rats challenged with 10 mg/kg LPS or with an equal volume of saline. The number of actively proliferating (BrdU positive) cells per crypt (15 crypts analyzed) in the terminal ileum was 0.14 ± 0.38 in LPS-treated rats compared with 4.45 ± 1.92 in saline-treated rats (P < 0.05). In the colon, the number of BrdU positive cells was 0.83 ± 0.43 in LPS-treated rats vs. 2.63 ± 0.52 in saline-injected rats (P < 0.05). This finding was observed at 16 and 24 h, but by 48 h after LPS challenge, there was no difference between the two groups (data not shown). These data suggest that in addition to inducing apoptosis, overproduction of NO or ONOO− can also blunt enterocyte proliferation in vivo.

Effect of ONOO− on enterocyte proliferation in vitro. To determine the mechanism by which ONOO− impairs enterocyte proliferation, we examined the effect of ONOO− on proliferation of the rat intestinal epithelial cell line, IEC-6, in vitro. The growth-arrested cells were cultured in the presence of buffer alone, ONOO−, or decomposed ONOO− for 5 min in media containing serum and [3H]thymidine, as described in MATERIALS AND METHODS. Figure 1 shows that ONOO− significantly inhibited proliferation of subconfluent monolayers of IEC-6 cells, as evidenced by a significant reduction in [3H]thymidine incorporation after treatment with authentic ONOO− compared with treatment with decomposed ONOO− or buffer alone. Because we have previously shown that exposure of IEC-6 cells to ONOO− for longer periods (>20 min) can induce apoptosis (33, 44), we measured IEC-6 apoptosis under the current experimental conditions to determine whether the decrease in proliferation was the result of increased apoptosis. Exposure of IEC-6 cells to 50 μM ONOO− for 1, 5, or 10 min followed by recovery in complete medium for 24 h had no significant effect on the percentage of IEC-6 apoptosis (data not shown).

To confirm that the observed effects of ONOO− on IEC-6 proliferation or apoptosis were due to nitrosative stress, we examined the ability of the NO donor, SNAP, and the superoxide generator, pyrogallol, to induce IEC-6 apoptosis alone or in combination. Increasing concentrations of SNAP had no effect on IEC-6 apoptosis, whereas pyrogallol (1 mM) led to a slight increase in IEC-6 apoptosis over baseline. However, the combi-
nation of SNAP plus pyrogallol, which produces ONOO\textsuperscript{-}, had a synergistic effect on IEC-6 apoptosis (Fig. 2). These results suggest that the effects of ONOO\textsuperscript{-} on IEC-6 apoptosis or proliferation may be due mainly to the nitrosative stress caused by ONOO\textsuperscript{-} itself rather than to oxidative injury.

Effect of ONOO\textsuperscript{-} on mitogen-induced proliferation of IEC-6 cells. To further define the mechanisms by which ONOO\textsuperscript{-} blunts the proliferation of IEC-6 cells in vitro, we examined whether ONOO\textsuperscript{-} could block proliferation induced by known mitogens such as FBS or EGF. We measured BrdU incorporation in serum-starved IEC-6 cells pretreated with ONOO\textsuperscript{-} or decomposed ONOO\textsuperscript{-} followed by exposure to FBS or EGF. As shown in Fig. 3, 48 h of serum starvation significantly suppressed IEC-6 proliferation. Neither ONOO\textsuperscript{-} nor decomposed ONOO\textsuperscript{-} had any significant effect on proliferation of serum-starved cells. The addition of PBS or EGF to the cultures partially restored IEC-6 proliferation. Pretreatment with 50 \( \mu \text{M} \) ONOO\textsuperscript{-}, but not decomposed ONOO\textsuperscript{-}, for 10 min before the addition of PBS or EGF resulted in a partial, but statistically significant, reduction in the proliferative response to these mitogens.

Effect of ONOO\textsuperscript{-} on Src kinase activity in IEC-6 cells. The Src family of nonreceptor tyrosine kinases plays an important role in enterocyte proliferation and signal transduction in response to growth factors. We hypothesized that ONOO\textsuperscript{-} may disrupt tyrosine kinase-dependent signaling pathways responsible for IEC-6 cell proliferation by nitrating critical tyrosine residues, thereby inhibiting Src phosphorylation. We examined the effect of ONOO\textsuperscript{-} on Src kinase activity in IEC-6 cells using an in vitro Src kinase phosphorylation assay. This assay determines the degree of Src activation by detecting the phosphorylation of immunoprecipitated Src. Src phosphorylation may be the result of autophosphorylation, or transphosphorylation by other immunoprecipitated Src molecules or by other coimmunoprecipitated proteins. Exposure to 50 \( \mu \text{M} \) ONOO\textsuperscript{-} resulted in a time-dependent decrease in Src phosphorylation (Fig. 4, top). Inhibition of Src kinase activity occurred as early as 1 min after ONOO\textsuperscript{-} treatment, but Src phosphorylation appeared to return to baseline levels by 30 min. There was no difference in the amount of Src protein among ONOO\textsuperscript{-}, decomposed ONOO\textsuperscript{-}, or buffer treatment groups as determined by Src immunoprecipitation followed by Western blot analysis by using anti-Src antibody (Fig. 4, bottom).
One potential mechanism for the inhibition of Src phosphorylation by ONOO⁻ is that ONOO⁻ may nitrate key tyrosine residues within Src, thus preventing phosphorylation of these residues. We therefore examined whether changes in the phosphorylation state of Src corresponding to tyrosine nitration of the protein occurred after exposure of IEC-6 cells to ONOO⁻. Protein lysates from the various treatment groups were immunoprecipitated with either anti-phosphotyrosine or anti-nitrotyrosine antibodies, with subsequent Western blot analysis with anti-Src antibody. There was a reduction in phosphorylated Src after a 1-min exposure to 50 μM ONOO⁻, but Src phosphorylation returned to baseline levels thereafter (Fig. 6A). This decrease in phosphorylation after 1 min was associated with a concomitant modest increase in tyrosine-nitrated Src after 1 min of ONOO⁻ treatment (Fig. 6B).

**Effect of ONOO⁻ on proliferation of v-Src-transfected enterocytes.** To further determine whether inhibition of Src by ONOO⁻ may be responsible for the decreased proliferative signaling by this kinase, we transfected IEC-6 cells with the constitutively active v-Src and measured proliferation in resulting transfectants after 48 h of serum starvation in the presence or absence of ONOO⁻. Figure 7 shows that ONOO⁻, but not decomposed ONOO⁻, blocked v-Src proliferative signaling although it was not as effective as the Src-specific inhibitor PP-1. Neither reagent had any effect on background [³H]thymidine incorporation in vector-transfected cells. This result is consistent with Src as a target of peroxynitrite in the proliferative response.

**Effect of ONOO⁻ on downstream targets of Src.** Activation of Src kinase by growth factors results in
phosphorylation of other downstream targets such as FAK that induce migration, differentiation or proliferation. We examined the effects of ONOO⁻ treatment on FAK phosphorylation in IEC-6 cells to determine whether this potential downstream target of Src is similarly affected by ONOO⁻ exposure. Protein lysates from IEC-6 cells treated with 50 μM ONOO⁻ or decomposed ONOO⁻ were immunoprecipitated with anti-phosphotyrosine antibody and analyzed by Western blot using an antibody to the Y576/577 region of FAK, which is a Src-dependent phosphorylation site. Exposure to authentic ONOO⁻ resulted in decreased phosphorylation at the Y576/577 site of FAK at 1 and 5 min (Fig. 8).

Activated Src has been demonstrated to migrate to focal adhesions and to bind FAK through a phosphotyrosine-SH2 interaction (30). We therefore examined whether ONOO⁻ exposure prevented interaction between Src and FAK. There were no differences in the amount of FAK immunoprecipitated with Src among the various groups (data not shown).

DISCUSSION

Sustained overproduction of NO has been demonstrated to occur in a variety of conditions characterized by gastrointestinal inflammation and gut barrier failure, such as endotoxemia, NEC, and IBD (4, 14, 26, 27, 40). ONOO⁻, a potent oxidant formed by the rapid reaction between NO and superoxide, is a key intermediate that is generated in inflammatory lesions in vivo and is generally thought to be responsible for pathological or cytotoxic effects of NO (3, 16, 21, 34, 35). Nitrotyrosine residues have been detected in multiple sites of inflammation and are generally thought to indicate the participation of reactive nitrogen intermediates, such as ONOO⁻, in the inflammatory process (3). In addition to direct nitration via ONOO⁻, ONOO⁻-independent tyrosine nitration may result from the reaction of hypochlorous acid (produced by neutrophils) with nitrite or via the reaction of nitrite with hydrogen peroxide under acidic conditions (13, 24). However, in our experiments, utilizing IEC-6 monolayers without neutrophils present and at neutral pH, direct tyrosine nitration by ONOO⁻ seems to be the most biologically feasible mechanism.

One mechanism by which ONOO⁻ can promote mucosal injury is by inducing enterocyte apoptosis (4, 14, 26–28, 33, 39, 40, 44). We (12) have previously shown that after endotoxemia in rats, enterocyte apoptosis colocalizes with iNOS protein upregulation and immunoreactivity to 3-nitrotyrosine. Scavenging NO or inhibiting iNOS ameliorates the derangement in epithelial barrier function after LPS challenge. However, in addition to enterocyte apoptosis, modulation of enterocyte proliferation in the intestinal crypts may also be a factor that contributes to mucosal injury and gut barrier failure (48). Impaired enterocyte proliferation and/or migration after endotoxemia may lead to an imbalance between mucosal injury and repair mechanisms, resulting in sustained gut barrier failure (32, 46). Thus inhibition of enterocyte proliferation may be an important unrecognized mechanism by which ONOO⁻ can induce and sustain mucosal injury and gut barrier failure.

Our data demonstrate that endotoxemia not only results in enterocyte apoptosis, but also leads to decreased enterocyte proliferation in the crypts, as evidenced by decreased BrdU incorporation in the intestinal epithelium. These observations suggest that overproduction of NO or ONOO⁻ can promote enterocyte apoptosis and reduce proliferation. It should be noted, however, that the effects of endotoxin are very complex and both proliferative and antiproliferative responses have been reported in the literature. For example, Rafferty et al. (36) found increased proliferation in the jejunal mucosa of rats challenged with 1 mg/kg LPS sq (twice) after 16 h. The differences between our results can be explained by the fact that we looked at the rat jejuna.
ileal mucosa of animals that received a much higher dose of LPS [10 mg/kg LPS ip (once)] at a later time point (24 h).

Analysis of intestinal mucosa from rats treated with LPS alone or with LPS plus the NO scavenger NOX (12) showed no difference in the mitotic index between the two groups despite the fact that there was significantly less epithelial damage and improved intestinal barrier function in the animals receiving LPS plus NOX (data not shown). This observation suggests that the inability to increase the rate of proliferation after epithelial injury may further exacerbate gut barrier dysfunction after endotoxemia. To further explore this hypothesis, we examined the effect of ONOO− on IEC-6 cells. ONOO− significantly reduced IEC-6 cell proliferation in vitro under experimental conditions that did not induce significant IEC-6 cell apoptosis over baseline levels (5–10 min); thus suggesting that the decrease in proliferation was not due to ONOO−-induced enterocyte cell death. Furthermore, ONOO− blocked the proliferative response of serum-starved IEC-6 cells to both FBS and EGF. These results strongly suggest that ONOO− may directly or indirectly inhibit proliferative cell signaling pathways in enterocytes. Although the reduction in the FBS and EGF responses were significant, the inhibition was not complete, suggesting that other proliferative pathways unaffected by ONOO− may also be activated in these cells on growth factor stimulation. Of note, the ability of ONOO− to inhibit proliferation occurred over the time course of 1 to 4 h used for our various experiments, despite the fact that the half-life of ONOO− is on the order of seconds (3). Thus ONOO− appears to induce cellular biochemical changes that persist despite the rapid decomposition of ONOO−. Nitration of key protein intermediates may be responsible for this effect, although we have not seen sustained nitration of Src or FAK throughout the time course of our experiments.

Proliferative signaling by EGF and other growth factors occurs through binding and autoprophosphorylation of receptor tyrosine kinases, which in turn may activate members of the Src family of nonreceptor tyrosine kinases (43, 45). The regulation and activation of Src are dependent on the phosphorylation state of two key tyrosine residues (38). Activated Src kinase then phosphorylates downstream targets such as Shc, phosphatidylinositol-3-kinase, and FAK, which then diverge along distinct signaling pathways that lead to cellular proliferation or differentiation (10, 17, 38).

The Src signaling pathway may be a potential site for modulation by ONOO− because ONOO− is known to nitrate tyrosine residues (3). Recent evidence suggests that ONOO− may interfere with tyrosine kinase signaling mechanisms. For example, ONOO− induced a dose-dependent inhibition of early protein tyrosine phosphorylation in activated lymphocytes and a corresponding increase in protein nitration (5). Also, ONOO− has been shown to modulate tyrosine phosphorylation and specifically, FAK phosphorylation in SH-SY5Y cells, a human neuroblastoma cell line (16, 22). In addition, Kong et al. (21) demonstrated that ONOO− nitrated a peptide derived from p34cdc2 kinase by Lyn, a Src family member. Interestingly, this tyrosine nitration event prevented the normal tyrosine phosphorylation of the p34cdc2 kinase peptide.

We have shown that ONOO− treatment can inhibit Src phosphorylation at early time points. Also, in addition to blocking Src activity in actively proliferating cells, ONOO− partially blocked FBS- and EGF-induced Src phosphorylation in serum-starved IEC-6 cells. Inhibition of Src phosphorylation by ONOO− was a transient event. This observation may be due to the extremely short half-life of ONOO− in neutral solution, which is on the order of seconds (3). It is possible that more sustained inhibition of Src may be observed in steady-state conditions associated with sustained or continual release or formation of ONOO−, as may occur in inflammatory conditions in vivo. The short-lived inhibition of Src phosphorylation suggests that ONOO− may also modulate proliferation via an unidentified mediator unrelated to Src, or that transient inhibition of Src can have long-lasting effects, possibly by modulation of downstream factors.

Although we speculate that ONOO−-induced Src nitration, decreased Src activity, and decreased entero-cyte proliferation are related, a direct causal relationship has not been established by our data. Indeed, the inhibition of Src phosphorylation, with only modest nitration of Src, suggests that other mechanisms besides tyrosine nitration may be responsible for inhibition of Src signaling. For example, ONOO− may affect upstream or downstream members of the Src signaling pathway independent of the effects on Src phosphorylation that we have demonstrated. In addition, it may be postulated that ONOO− may interfere with Src signaling via its oxidant properties by affecting the redox state of the local cellular milieu. However, previous reports have suggested that oxidants primarily induce cellular proliferation and, in particular, activate the Src family of tyrosine kinases (1, 2, 11, 19). In contrast, we showed that neither the superoxide donor, pyrogallol, nor the NO donor, SNAP, induced significant IEC-6 apoptosis when added alone, although they had a synergistic effect on IEC-6 apoptosis when added together, presumably by forming ONOO− in the cultures. These data support our hypothesis that ONOO− inhibits enterocyte proliferation and Src kinase activity via nitrosative stress and tyrosine nitration rather than through its oxidant properties. Furthermore, our data show that ONOO− blocked proliferative signaling in IEC-6 cells transfected with the constitutively active v-Src and inhibited Src-dependent phosphorylation of the downstream target FAK, which is a kinase important in promoting cell attachment, motility, and survival (30, 38). This inhibition of FAK phosphorylation occurred without inhibition of Src-FAK interaction, as ONOO− did not affect communoprecipitation of FAK with Src. Because FAK is a key protein in the regulation of cell spreading, migration, survival, and formation of cell-cell junctions, inhibition of FAK by ONOO− may lead to inhibition of key cellular processes re-
quired for maintenance of epithelial integrity and repair of mucosal injury.

There have been somewhat conflicting results concerning the effect of ONOO\(^-\) on Src kinase activity. A number of studies have shown both nitration and activation of Src by ONOO\(^-\) (20, 23, 25). These experiments utilized a variety of cell types and generally showed increased Src activity only with high concentrations of ONOO\(^-\) (500 to 1,000 \(\mu\)M) which is 10 to 20 times higher than the dose (50 \(\mu\)M) used in our experiments (20, 23). In contrast, Mallozzi et al. (25) demonstrated that ONOO\(^-\), at low concentrations, activated Src kinase activity, whereas higher concentrations of ONOO\(^-\) (>250 \(\mu\)M) inhibited activity of Src kinase family members. Thus the effect of ONOO\(^-\) on Src activity may be concentration dependent and cell-type dependent. In contrast to these previous reports, we also correlated our Src kinase activity results with corresponding changes in cellular proliferation.

Although there are estimates of the rate of ONOO\(^-\) production by isolated alveolar macrophages in vitro, the complex biochemistry and short half-life of ONOO\(^-\) make it difficult to measure rates of intestinal mucosal ONOO\(^-\) production during inflammation (18). In addition, because of the short half-life and resultant limited diffusion of ONOO\(^-\), the local concentration of ONOO\(^-\) to which any specific enterocyte within the intestinal mucosa will be exposed may vary widely. Thus the choice of ONOO\(^-\) concentrations to use for experiments is largely empirical. We have used a concentration of ONOO\(^-\) (50 \(\mu\)M), which is significantly lower than that used by other investigators in different cell types, because the lower concentrations of ONOO\(^-\) are more likely to be physiologically relevant. In addition, we developed a treatment regimen that did not induce apoptosis in our IEC-6 cells and therefore did not confound the effects of ONOO\(^-\) on proliferation and cell signaling pathways.

In summary, we have shown that ONOO\(^-\) inhibits proliferation and blocks the proliferative response to FBS, EGF, and v-Src transfection in an enterocyte cell line. These events are associated with decreased Src and FAK phosphorylation. Thus in addition to causing mucosal injury by inducing enterocyte apoptosis, ONOO\(^-\) can contribute to mucosal inflammation and gut barrier dysfunction by inhibiting enterocyte proliferation via the Src kinase signaling pathway.

DISCLOSURES

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REFERENCES


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