Relative contributions of NOS isoforms during experimental colitis: endothelial-derived NOS maintains mucosal integrity

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Submitted 26 April 2004; accepted in final form 16 June 2004

Vallance, Bruce A., Gerard Dijkstra, Bosheng Qiu, Laurens A. van der Waaaij, Harry van Goor, Peter L. M. Jansen, Hiroshi Mashimo, and Stephen M. Collins. Relative contributions of NOS isoforms during experimental colitis: endothelial-derived NOS maintains mucosal integrity. *Am J Physiol Gastrointest Liver Physiol* 287: G865–G874, 2004. First published June 24, 2004; 10.1152/ajpgi.00187.2004.—The role of nitric oxide (NO) in inflammatory bowel diseases has traditionally focused on the inducible form of NO synthase (iNOS). However, the constitutive endothelial (eNOS) and neuronal (nNOS) isoforms may also impact on colitis, either by contributing to the inflammation or by regulating mucosal integrity in response to noxious stimuli. To date, studies examining the roles of the NOS isoforms in experimental colitis have been conflicting, and the mechanisms by which these enzymes exert their effects remain unclear. To investigate and clarify the roles of the NOS isoforms in gut inflammation, we induced trinitrobenzenesulfonic acid colitis in eNOS, nNOS, and iNOS knockout (KO) mice, assessing the course of colitis at early and late times. Both eNOS and iNOS KO mice developed a more severe colitis compared with wild-type mice. During colitis, iNOS expression dramatically increased on epithelial and lamina propria mononuclear cells, whereas eNOS expression remained localized to endothelial cells. Electron and fluorescence microscopy identified bacteria in the ulcerated colonic mucosa of eNOS KO mice, but not in wild-type, iNOS, or nNOS KO mice. Furthermore, eNOS KO mice had fewer colonic goblet cells, impaired mucin production, and exhibited increased susceptibility to an inflammatory stimulus that was subthreshold to other mice. This susceptibility was reversible, because the NO donor isosorbide dinitrate normalized goblet cell numbers and ameliorated subsequent colitis in eNOS KO mice. These results identify a protective role for both iNOS and eNOS during colitis, with eNOS deficiency resulting in impaired intestinal defense against luminal bacteria and increased susceptibility to colitis.

SEVERAL FACTORS HAVE BEEN implicated in the pathogenesis of human inflammatory bowel diseases (IBD) including an immunological intolerance to enteric microflora (10–12, 43), as well as defects in mucosal barrier function (47). Recently, attention has been focused on the overproduction of nitric oxide (NO) in IBD (7, 31, 48). Several studies have identified increased levels of NO in the rectal dialysates (46), in the inflamed mucosa of patients with ulcerative colitis (UC) (3), and in animal models of colitis (26, 37, 60). The increased NO synthase (NOS) activity was identified predominantly as the inducible form of NOS (iNOS) (23). Whereas the two constitutive isoforms, neuronal (nNOS) and endothelial-derived (eNOS), modulate several aspects of intestinal physiology (6, 53), their contribution to the inflammatory response has received less attention.

Despite considerable evidence that iNOS-derived NO contributes to tissue destruction in colitis and other inflammatory states (27), conflicting results have been obtained by using pharmacological inhibitors of NOS in animal models of IBD. Several studies have found that the nonspecific NO inhibitor Nω-nitro-L-arginine methyl ester reduced intestinal inflammation (17, 37, 45); other studies found little benefit (20) or enhancement of colitis (42). Similar disparate results have been obtained by using more selective iNOS inhibitors (54, 61). Whereas these findings may reflect differences in dosing regimens, they may also reflect the consequences of inhibiting not only iNOS, but also the constitutive forms of NOS. These limitations have encouraged the study of colitis in mice genetically deficient in iNOS. Unfortunately, these studies (34, 35, 62) have utilized different protocols and yielded conflicting results. Moreover, the few studies that have examined the roles of the other NOS isoforms in experimental colitis have also been conflicting; such that it remains unclear whether the eNOS and nNOS isoforms contribute to intestinal inflammation (2, 16) or whether they instead maintain intestinal homeostasis and mucosal integrity (5, 48).

In this study, we examined the role of each of the three NOS isoforms in experimental colitis by using mice genetically deficient in iNOS (32), nNOS (21), or eNOS (22). Colitis was induced by intrarectal administration of trinitrobenzenesulfonic acid (TNBS) dissolved in 50% ethanol as a mucosal barrier breaker. Our results indicate that both iNOS and eNOS knock-out (KO) mice develop an exaggerated and prolonged colitis. The absence of eNOS reduced the integrity of the colonic mucosa, as reflected by fewer goblet cells, and the production of less mucin in colonic tissues removed from eNOS KO mice. Moreover, only eNOS KO mice developed colitis using TNBS in 30% ethanol as a barrier breaker, illustrating their increased susceptibility to mucosal injury. Whereas these results confirm the protective role of iNOS, they also generate the novel concept that eNOS is critical for colonic mucosal integrity by maintaining goblet cell numbers and function and thereby

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protecting against inflammatory stimuli and bacterial translocation.

MATERIALS AND METHODS

Mice. Specific pathogen-free, male C57BL/6 mice (6 to 10 wk old) (Taconic Farms, Germantown, NY), and 129/J mice (Jackson Laboratories, Bar Harbor, ME) were used as wild-type mice. iNOS KO mice (on C57BL/6 background) (32) were also purchased from Jackson Laboratories. The eNOS (22) and nNOS (21) KO mice (mixed C57BL/6 × 129/J background) were obtained from Massachusetts General Hospital, Harvard Medical School (from H. Mashimo). Because the eNOS and nNOS KO mice were on a mixed background, we assessed both background strains as controls. Experimental protocols followed McMaster University and the University of British Columbia’s Animal Care Committees and the Canadian Council on the Use of Laboratory Animals guidelines.

Colitis induction and assessment. Mice were anesthetized with Enflurane, (Abbott Laboratories, St. Laurent, PQ, Canada) and given an intrarectal enema of 6 mg of trinitrobenzenesulfonic acid (TNBS) (Eastman Kodak, Rochester, NY) in 50% ethanol (vol/vol) (diluted in dH2O) using a catheter made of polyethylene tubing (PE-50) attached to a 1-ml syringe as described previously (52). Colonic damage was assessed as previously described (44, 52), and a total cumulative score was expressed as the ulcer index (UI).

MPO activity. Mucosal MPO activity was measured by colorimetric assay as described previously (29). In brief, the colonic mucosa was scraped from the underlying muscularis and weighed. The tissue was homogenized in phosphate buffer (pH 7.4) containing hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO). Homogenates were centrifuged at 15,000 rpm for 20 min, and the supernatant was mixed with o-dianisidine (Sigma) and hydrogen peroxide reagent (Caledon Laboratories, Georgetown, ON, Canada). Samples were measured at 560 nm with MPO activity expressed as units per gram of protein. Protein levels were estimated by the BCA protein assay (Pierce, Rockford, IL).

Measurement of plasma nitrite levels. Plasma nitrite levels were measured by spectrophotometer on the basis of the Griess reaction. Briefly, blood samples were centrifuged, and the supernatant was aspirated off. Blood samples were mixed in 96-well microtiter plates with Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, and 25% H3PO4; Sigma). After 10 min, the optical density was measured at 595 nm.

NO restoration. The NO donor isosorbide dinitrate (Sigma) was administered intrarectally to eNOS KO mice in a dose of 20 mg/kg every 8 h, three times per day. After 3 days, colitis was induced, or administered intrarectally to eNOS KO mice in a dose of 20 mg/kg.

Histology. Immediately after death, colonic tissues were opened longitudinally, cleaned, and fixed in 10% formalin overnight. Tissues were paraffin embedded, if both observers agreed.

Fluorescence in situ hybridization. Cryosections (4 μm) were fixed in 4% paraformaldehyde for 24 h at 4°C. Sections were hybridized overnight in 10 μl of hybridization buffer containing a FITC-labeled Bact338 probe (reacting with most bacteria) (13) at a concentration of 5 ng/μl. Sections were washed for 20 min at 50°C in hybridization buffer without SDS. Slides were then dipped in distilled water, dried, and mounted by using Vectashield (Vector Laboratories, Burlingame, CA). Two people evaluated each section using a fluorescent microscope. Particles were considered bacteria (seen as a bright green rods and coccooids against a yellow-brown autofluorescence background), if both observers agreed.

Bacterial counts. To assess whether the NOs deficiencies impacted on bacterial translocation out of the colon under normal or colitic conditions, mice were killed and their mesenteric lymph nodes (MLN) were removed. The MLN were then homogenized at a low speed using a Kinematica tissue homogenizer (Brinkmann) as previously described (56). Homogenates were serially diluted and plated onto Luria broth plates and incubated at 37°C overnight. Bacterial colonies were then enumerated the following day. Ten bacterial colony forming units per mouse was the minimum number of bacteria that could be identified by this approach.

Electron microscopy. Colon segments were removed and fixed with 2% glutaraldehyde for 2 h and postfixed with 1% osmium tetroxide for 1 h. Tissues were dehydrated through an alcohol gradient and embedded in Epon. Ultrathin sections were cut at a thickness of 90 nm and observed with a transmission electron microscope (JEM-1200 EX).

Measurement of colonic mucin glycoprotein release. Colonic mucosal explants (2 × 2-mm size) were cultured in 35-mm tissue culture dishes (Becton Dickinson Labware) in 1.5-ml Trowell’s medium containing 10 μCi/ml of [3H]glucosamine (DuPont-New England Nuclear) in the presence of antibiotic-antimycotics (Life Technologies). [3H]Glucosamine incorporation into glycoproteins was determined as previously described (4) and quantified as disintegrations per minute of [3H]glucosamine per gram protein. To confirm [3H]glucosamine was incorporated into mucin, the TCA/PTA precipitate was examined by density gradient ultra centrifugation as described previously (4).

Data presentation and statistical analysis. For most experiments, 8–10 mice per group were tested. Results are expressed as the means ± SE. Statistical significance was calculated by using the Student’s t-test for comparison of two means or a one-way ANOVA for the comparison of three or more means. Multiple comparisons were performed by using the Newman-Keuls multiple comparison test. P < 0.05 was considered significant.

RESULTS

Colitis in NOS isoform KO mice. We first examined plasma nitrite levels in NOS isoform KO and wild-type mice before colitis. Circulating nitrite levels were similar in the iNOS KO, nNOS KO, and wild-type mice (4.5 to 4.9 μM) (Table 1), but...
Nitrite levels were significantly lower ($P < 0.01$) in the eNOS KO mice, at $0.9 \pm 0.1 \mu$M, identifying eNOS as the major source of circulating NO under basal conditions.

We then examined the susceptibility of NOS KO mice to experimental colitis. Whereas colitis did not significantly increase nitrite levels in iNOS KO mice ($5.1 \pm 0.7 \mu$M) by day 3, nitrite levels rose significantly in all other mouse strains tested ($14.6$ to $16.0 \mu$M) (see Table 1). Impressive macroscopic damage was seen by day 3 in the nNOS KO mice (UI, $7.8 \pm 1.1$) and the iNOS KO mice (UI, $9.1 \pm 1.0$) with UI values similar to wild-type C57BL/6 (UI, $8.6 \pm 0.8$), and 129/J mice (UI, $8.3 \pm 1.6$) with colitis. However, eNOS KO mice exhibited more extensive and severe colitis with a significantly higher UI ($12.3 \pm 1.3$, $P < 0.05$) compared with wild-type strains (see Fig. 1, A and B). Granulocytic infiltration, as assessed by MPO activity, was also similar in the nNOS KO ($17.0 \pm 2.9$ U/g), C57BL/6 ($17.5 \pm 2.2$ U/g), and 129/J mice ($17.0 \pm 3.5$ U/g). However, MPO activity was significantly higher in both the eNOS ($37.5 \pm 2.2$ U/g, $P < 0.01$) and the iNOS KO mice ($30.0 \pm 6.6$ U/g, $P < 0.05$).

To determine the influence of NO on the duration of colitis, we examined NOS KO mice at day 14 postcolitis. Whereas most damage had healed in the nNOS KO (UI 2.7 $\pm 0.6$, MPO 12.0 $\pm 1.1$ U/g), C57BL/6 (UI 1.8 $\pm 0.3$, MPO 10.0 $\pm 0.9$ U/g), and 129/J mice (UI 1.6 $\pm 0.4$, MPO 9.6 $\pm 2.2$ U/g), tissue injury and inflammation were still evident in the eNOS KO mice (UI 3.4 $\pm 0.7$, MPO 18.0 $\pm 2.0$ U/g), and the iNOS KO mice (UI 3.5 $\pm 0.5$, MPO 20.0 $\pm 2.1$ U/g) with all inflammatory markers significantly higher than in wild-type mice ($P < 0.05$). Thus eNOS and iNOS isoforms not only protect against an initial colitis injury but also influence the duration of the resulting tissue damage and inflammation in the colonic mucosa.

**Immunostaining for iNOS and eNOS.** Because our studies identified the iNOS and eNOS isoforms as impacting on the severity of TNBS colitis, we examined the expression of these isoforms within the colon by immunohistochemistry. Little iNOS expression was seen in the colons of wild-type mice without colitis (Fig. 2A) but strong iNOS expression was seen on colonocytes and lamina propria mononuclear cells during...
colitis (Fig. 2B). iNOS staining in the nNOS KO mice was seen only during colitis and was similar to that seen in wild-type mice (not shown). In contrast, moderate staining for iNOS protein was seen in epithelial and mononuclear cells in eNOS KO mice, even in the absence of colitis, but the iNOS staining was limited to sites adjacent to lymphoid follicles (not shown). As shown in Fig. 2C, iNOS staining dramatically increased in the eNOS KO mice during colitis. As expected, iNOS KO mice did not express immunoreactive iNOS protein under any condition (Fig. 2D).

In wild-type mice, eNOS expression was limited to endothelial cells lining colonic blood vessels (Fig. 2E), and the location of eNOS expression did not change after induction of colitis. However, the size and number of blood vessels, and thus eNOS expression, did increase during colitis, particularly within the lamina propria, (Fig. 2F). A similar pattern of eNOS expression was observed in the nNOS and iNOS KO mice, and the size and number of eNOS staining blood vessels increased during colitis in these mouse strains as well. No eNOS expression was detected in the eNOS KO mice under any condition (not shown).

Immunostaining for nitrotyrosine. One cytotoxic mechanism of NO is through its interaction with superoxide to produce peroxynitrite, which causes the nitration of amino acid residues such as tyrosine. Whereas NO has a short half-life, nitrotyrosine epitopes are stable and tend to accumulate in inflammatory cells. Nitrotyrosine-positive cells were rare in the uninflamed colon from all mouse strains (see Fig. 3A). During colitis, nitrotyrosine staining was found predominantly in lamina propria mononuclear cells and more of these cells stained positively for nitrotyrosine than for iNOS. Interestingly, no nitrotyrosine staining was seen on epithelial cells during colitis (Fig. 3B). Endothelial cells were also negative for nitrotyrosine. Whereas all mouse strains including the iNOS KO mice (Fig. 3D) showed increased levels of nitrotyrosine staining during colitis, the eNOS KO mice (Fig. 3C) showed the greatest numbers of positively staining cells. The former finding implies that both iNOS-dependent and -independent path-
ways leading to the formation of nitrotyrosine are active during TNBS colitis, (30, 38).

**Bacterial translocation.** In view of the important role that colonic microflora play in the TNBS colitis model (15), we examined colonic mucosal tissues for bacteria using electron microscopy and fluorescence in situ hybridization, using commercial probes that recognize the 16S RNA of most commensal enteric bacteria (13). Whereas bacteria were occasionally identified within the adherent mucus layer overlying the colonic epithelium, no bacteria were seen within the mucosa of control mice (Fig. 4A).

Despite the mucosal ulceration induced by TNBS, no overt bacterial penetration of the mucosal barrier was seen in wild-type, nNOS, or iNOS KO mice (Fig. 4B). In contrast, large numbers of bacteria were identified just beneath ulcerated areas in the eNOS KO mice. As seen in Fig. 4C, fluorescing bacteria appeared to be translocating across the colonic mucosa. The presence of bacteria within the colonic mucosa of colitic eNOS KO mice was confirmed by electron microscopy (see Fig. 4D).

To determine whether the bacteria seen in the colonic mucosa of colitic eNOS KO mice reflected an increased susceptibility of these mice to bacterial translocation from the colon, we quantified bacterial numbers in the MLN of both wild-type and NOS KO mice with or without colitis. No bacterial colonies were isolated from noncolitic mice, but at day 3 postcolitis, small numbers of bacteria (ranging from 0 to 640) were found in the MLNs of colitic mice of all genotypes (Table 2). However, a significantly greater number of bacteria ($1.6 \pm 0.8 \times 10^3$) were found in the MLNs of colitic eNOS KO mice.
**Susceptibility to colitis.** To determine whether eNOS and iNOS KO mice suffered from an inability to downregulate the inflammatory response or from an increased susceptibility to the initial insult, we administered TNBS in only 30% ethanol (rather than the normal 50%). As shown in Table 3, little damage was seen in wild-type, nNOS, or iNOS KO mice. In contrast, eNOS KO mice developed significant ulceration ($P < 0.05$) and MPO activity ($P < 0.05$). These results indicate that the exaggerated colitis seen in eNOS KO mice reflects an increased susceptibility to the initial insult.

**Goblet cell numbers and function.** To further pursue the mechanisms underlying the increased susceptibility of eNOS KO mice to colitis, histological analysis identified a significant reduction in the number of superficial PAS-positive goblet cells per 100 colonocytes in the distal colon of eNOS KO mice (5.4 ± 1.0) compared with control C57BL/6 mice (14.2 ± 1.7, $P < 0.001$) and 129/J mice (12.7 ± 2.3, $P < 0.005$) (Fig. 5). In contrast, nNOS KO and iNOS KO mice had numbers of goblet cells (12.8 ± 1.7 and 15.2 ± 1.8, respectively) similar to wild-type numbers.

Electron microscopy revealed that goblet cells in eNOS KO mice were smaller and contained less mucin than in wild-type mice (see Fig. 5). Moreover, mucin release from eNOS KO mice were smaller and contained less mucin than in wild-type mice (4.1 ± 0.8 dpm of $[^3]$H]glucosamine/g protein vs. 11.5 ± 1.4 dpm, $P < 0.001$) as well as iNOS and nNOS KO mice (10.4 ± 1.6 and 12.0 ± 1.6 dpm/g, respectively). Because studies in colitic eNOS KO mice found that goblet cell numbers significantly increased by day 3 post-TNBS (18.1 ± 1.7, $P < 0.001$), we reasoned that the eNOS KO goblet cell deficiency reflected a potentially reversible response to reduced NO production, rather than a development of the NO donor isosorbide nitrate. Treatment over 3 days attenuated. Our findings are similar to those seen in collagenous colitis (41), where high iNOS expression by intestinal epithelial cells is not associated with overt mucosal tissue damage. In fact, like McCafferty et al. (34, 35) we found that iNOS KO mice exhibited a more severe and protracted colitis, suggesting that rather than acting as a noxious substance during inflammation, NO may have beneficial actions. The absence of iNOS expression under control conditions suggests that iNOS plays its beneficial role after the induction of colitis. Indeed, we found no evidence that iNOS deficiency altered the susceptibility of the colon to an inflammatory insult. However, iNOS appeared to play its role at the later stages of infection, because although iNOS KO mice showed a similar degree of colitis as wild-type mice at day 3, they were impaired in their ability to resolve the inflammation and tissue damage seen at day 14 postcolitis. The counterinflammatory effects of iNOS-derived NO are poorly understood but may involve the maintenance mucosal perfusion, the prevention of mast cell activation, and a reduction in leukocyte adherence to endothelium (30).

**DISCUSSION**

iNOS is believed to represent the major source of NO in IBD (26). Histological studies have identified intense focal iNOS expression by the inflamed bowel epithelium and in the mononuclear cell infiltrate in the intestinal tissues of both Crohn’s disease and UC patients (9, 50). We therefore reasoned that iNOS would play a pivotal role in experimental intestinal inflammation. Indeed, our data suggest that NO produced by iNOS is the major source of the increased plasma nitrite seen during experimental colitis. iNOS expression strongly increased during TNBS colitis and, as seen in UC patient tissues (9, 24), was expressed predominantly by epithelial cells at sites proximal to inflammatory infiltrates, as well as by lamina propria mononuclear cells. However, in the absence of iNOS expression, the tissue damage seen in this model was not attenuated. Our findings are similar to those seen in collagenous colitis (41), where high iNOS expression by intestinal epithelial cells is not associated with overt mucosal tissue damage.

**Table 2. Bacterial translocation to MLN**

<table>
<thead>
<tr>
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<th>C57BL/6</th>
<th>129/J</th>
<th>nNOS KO</th>
<th>iNOS KO</th>
<th>eNOS KO</th>
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<tr>
<td>CFU/mouse day 0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
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<tr>
<td>CFU/mouse day 3</td>
<td>1.6 ± 0.4 × 10^2</td>
<td>0.9 ± 0.2 × 10^2</td>
<td>1.0 ± 0.2 × 10^2</td>
<td>2.2 ± 0.5 × 10^2</td>
<td>1.6 ± 0.8 × 10^3</td>
</tr>
</tbody>
</table>

Values are mean colony forming units (CFU) of bacteria recovered from the mesenteric lymph nodes (MLN) of mice ± 1 SE for groups of 5–6 mice.

**Table 3. Colitis in response to 30% ethanol**

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>129/J</th>
<th>nNOS KO</th>
<th>iNOS KO</th>
<th>eNOS KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulcer index</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>1.1 ± 0.3</td>
<td>4.0 ± 0.3*</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>10.2 ± 1.2</td>
<td>9.7 ± 1.5</td>
<td>8.1 ± 1.1</td>
<td>7.6 ± 1.5</td>
<td>20.0 ± 2.0*</td>
</tr>
</tbody>
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Values are in arbitrary units for ulcer index and in mean units of activity per gram of protein for myeloperoxidase ± 1 SE for groups of 8–10 mice.

* Significant difference from both C57BL/6 and 129/J values ($P < 0.05$).
iNOS expression by cultured enterocytes is accompanied by apical NO release (28), epithelial-derived NO may also function in an antimicrobial role against enteric bacteria. This hypothesis is supported by studies in which enteroinvasive bacteria induce iNOS expression by cultured epithelial cells (59), similar to the effects of several proinflammatory cytokines (28). Moreover, we recently demonstrated that iNOS is strongly induced on colonic epithelial cells in mice infected with the gram-negative bacterial pathogen *Citrobacter rodentium*, a mouse-adapted version of enteropathogenic *Escherichia coli*. In that earlier study (56), iNOS-deficient mice suffered a worsened colitis and a prolonged infection, indicating that epithelial-derived iNOS does play a role in protecting the host against colonic bacteria.

The actions of nNOS have been studied in many systems, including in the airways in which its actions are crucial to the development of some asthma models (8). Less is known about the role of nNOS in experimental colitis. Studies have shown that intestinal inflammation disrupts NADPH diaphorase staining in gut tissues (20), whereas nNOS expression by enteric nerves is altered in UC patient tissues (57). These observations suggest colitis affects both NO-based neurotransmission and gastrointestinal motility (39, 53). Beck et al. (2) using the DSS colitis model, recently identified nNOS as playing an important role in that model, with the loss of nNOS expression resulting in more severe disease and increased mortality. The protective mechanisms exerted by nNOS in that study were not defined, and in contrast, our study indicates that nNOS does not play a significant role in the generation of the inflammatory response induced by TNBS in the mouse. The divergent results between this study and the work by Beck et al. (2) likely reflect the different colitis models used.

Two studies have previously examined the susceptibility of eNOS KO mice to experimental colitis. Beck et al. (2) found that mice deficient in eNOS or iNOS suffered less tissue damage and inflammation than wild-type mice during DSS-
induced colitis. In contrast, Sasaki et al. (48) showed that eNOS was protective using the same model. Our data also support a protective role for eNOS in experimental colitis. Moreover, our results indicate that eNOS is the major contributor to circulating nitrite levels under normal conditions. Both before and during colitis, eNOS expression was restricted to colonic vascular endothelium, as has been found in IBD tissues (9). In the absence of eNOS, basal plasma nitrite levels were reduced. However, during colitis, nitrite levels in eNOS KO mice increased to levels similar to those seen in wild-type colitic mice, likely reflecting NO from iNOS origin. Indeed, iNOS expression was readily detected in tissues from colitic eNOS KO mice, indicating that eNOS deficiency did not interfere with iNOS expression.

We also examined the mechanisms underlying the protective role of eNOS, finding that eNOS plays a major role in protection against TNBS-induced colitis and bacterial translocation. In this model, 50% ethanol acts as a mucosal barrier breaker, facilitating the entry of the haptenizing agent TNBS into the colonic mucosa where it modifies host proteins. Unlike the other mouse strains tested, eNOS KO mice developed significant colitis in response to TNBS dissolved in 30% ethanol, demonstrating a weakened mucosal barrier we attribute to a striking deficit in goblet cell numbers and mucin production. Several studies (1, 40, 51) have implicated goblet cells and mucus production as important factors protecting the colon against injury, whereas others (18, 55) have identified abnormalities in mucin production in IBD. Unfortunately, it is unclear whether these changes are causal or are secondary to the disease process.

Our study also indicates that eNOS deficiency not only increased susceptibility to normally noncolitic stimuli, but that the eNOS-dependent goblet cell deficiency was a contributing factor to the exaggerated colitic response seen in the eNOS KO mice. After the normalization of goblet cell numbers by locally administering a NO donor to the colon, subsequent colitis was significantly attenuated. Whereas a direct effect of NO on goblet cells is possible, eNOS has been traditionally thought to act as a vasodilator. Therefore, defects in eNOS expression may impair blood flow to the mucosa, resulting in poor growth and function of goblet cells, and susceptibility to intestinal inflammation. Whereas the evidence that a primary defect in eNOS expression plays a major role in IBD is sparse, a preliminary study did show that early onset of UC in a Japanese population was associated with polymorphisms of the eNOS gene (14). Alternatively, an eNOS dysfunction in IBD could be acquired as a result of the chronic inflammation seen in these diseases. This hypothesis has been substantiated by a recent study by Hatoum et al. (19) that identified an acquired microvascular dysfunction in IBD tissues, resulting in a loss of NO-mediated vasodilation in the inflamed mucosa. Our findings that eNOS plays an important role in maintaining mucosal integrity in the GI tract are intriguing; however, more studies are needed to assess whether alterations in eNOS expression or function contribute to the pathogenesis of IBD.

Identification of significant numbers of microorganisms near ulcerated areas of the colon and in the MLNs of eNOS KO mice also leads us to conclude that eNOS-derived NO plays an important role in defense against bacterial translocation. This may be due to the impaired goblet cell growth and mucin production seen in these mice. Unlike the IL-10 and IL-2
gene-deficient mouse models in which defects in immune regulation result in spontaneous colitis, if the animals are kept under conventional but not germfree housing (47), none of the NOS KO strains showed any overt signs of spontaneous intestinal inflammation in this study. We therefore propose that under homeostatic conditions, the mucosal barrier of the iNOS and eNOS KO mice provides adequate protection against luminal products, with exaggerated inflammation and bacterial translocation seen only when this mucosal barrier is challenged. In addition, it may reflect the actions of eNOS-derived NO in mediating the vascular relaxation seen during inflammation (49) such that the loss of eNOS may prevent the mucosal immune system from effectively dealing with the translocated bacteria. Given the importance of enteric microflora and epithelial barrier function in IBD, it is tempting to speculate that eNOS-derived NO may play an important role in the pathogenesis of IBD.

In summary, these results demonstrate that eNOS plays an important role in mediating intestinal homeostasis and mucosal integrity when the colon is challenged by using the TNBS colitis model. These studies also provide the novel discovery of eNOS involvement in goblet cell function and growth in the colon, providing a likely mechanism by which eNOS KO mice are more susceptible to experimental colitis and bacterial translocation. Further studies examining the potential role of eNOS and goblet cells in the pathogenesis of IBD are recommended, because our studies found that local NO donors were able to normalize colonic defenses in eNOS KO mice. If defects in eNOS expression do occur in IBD, these studies suggest that delivering NO donors could prove an effective approach to maintain intestinal homeostasis in the face of potential noxious stimuli that could trigger IBD or other gastrointestinal diseases.

ACKNOWLEDGMENTS

The authors thank Trish Blennerhassett, Alie de Jager-Krikken, and Ryan Barrett for technical expertise and Yvonne Chan for her expert administrative assistance.

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