eNOS involved in colitis-induced mucosal blood flow increase

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Submitted 8 August 2007; accepted in final form 17 October 2007

NITRIC OXIDE (NO) HAS BEEN shown to be involved in the pathogenesis of the inflammatory bowel diseases (IBD) ulcerative colitis and Crohn’s disease (18, 24, 55). However, the role of NO in IBD is still controversial, since some studies suggest that NO has protective influence, whereas others indicate a pathogenic role [reviewed by Kolios and colleagues (18)]. Recently, high levels of luminal NO have been found during intestinal inflammation (28–31, 44, 50), highlighting the association of NO with IBD.

The involvement of NO in blood flow regulation in the upper gastrointestinal tract and the importance of gastric mucosal blood flow in the defense against injury to the gastric mucosa have been well studied (1, 17). The regulation of colonic mucosal blood flow is poorly understood, and its importance during colonic injury is still unclear. Studies in recent years have yielded contradictory data, showing either an increased (8, 11, 14, 27) or a decreased (7, 11, 21, 22, 38) blood flow in different animal models of colitis, using a variety of measuring techniques. The few blood flow studies conducted in humans with IBD have indicated that the mucosal blood flow is increased in inflamed regions (3, 15).

A well-regulated colonic mucosal blood flow is of utmost importance for sustained homeostasis. Epithelial integrity is dependent on mucosal blood flow for delivering oxygen, nutrients, and leukocytes when required and for removing reactive oxygen species and intruding bacteria (32). During periods of ischemia, the colonic barrier immediately breaks down, a condition which, if left untreated, will lead to sepsis.

In the colon, mucosal capillaries normally form a regular honeycomb-like plexus around the opening of the mucosal glands (53), and this colonic microvasculature becomes dilated during colitis (10, 34, 45, 48). NO formed in endothelial cells plays an important part in the regulation of the regional blood flow. Endothelial nitric oxide synthase (eNOS) produces NO that dilates the submucosal arterioles, leading to increased blood flow in the mucosal capillaries (55). In mice deficient in eNOS, the inflammation induced in different models of colitis is more severe, indicating that eNOS has an important protective role against mucosal inflammation (51, 56).

Although NO is involved in many physiological functions, it also contributes to tissue injury during inflammatory processes. When produced in small amounts, NO generally exerts positive effects in the gastrointestinal tract (55). However, under inflammatory colonic conditions, greatly increased NO levels have been reported, for example, in human IBD (47, 49) and in animal models of colitis (26). Under these conditions, NO is produced in large amounts mainly by inducible NO (iNOS), and it has been proposed that this high NO level causes injury through formation of peroxynitrite (24). Expression of iNOS is induced during inflammation, and hence iNOS is highly up-regulated in inflamed colonic mucosa (4, 33, 45). iNOS is primarily expressed in macrophages and neutrophils (55), but during colitis it is also expressed in large amounts by epithelial cells (35, 45). Interestingly, induction of experimental colitis in iNOS-deficient mice results in less severe inflammation (4, 20, 59) in contrast to the findings in mice with eNOS deficiency.

Neuronal NO (nNOS) in the colonic myenteric plexus has been shown to play an important part in the regulation of colonic peristalsis and transit (54), and there are data indicating...
that loss of nNOS during colonic inflammation results in dysfunction of colonic peristalsis (36).

The aim of this study was to measure colonic mucosal blood flow both in untreated rats and in rats with colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) or dextran sulfate sodium (DSS). Furthermore, since large amounts of NO are present in the colon during colitis (28, 29, 31, 44, 50), a further purpose was to determine whether NO is involved in the regulation of colonic mucosal blood flow in colitis and in control animals. An attempt was also made to ascertain which of the NOS isoforms is most important in blood flow regulation.

MATERIALS AND METHODS

Animal preparation. All experiments were approved by the regional ethics committee for animal experiments in Uppsala.

Male Sprague-Dawley rats (B&K, Sollentuna; Sverige and Møllegaard Breeding Center, Ejby, Denmark), weighing between 190 and 290 g (weight before treatment), were kept under standardized conditions at a temperature of 21–22°C and with 12 h light and 12 h dark per day. They were allowed to acclimatize for 1 wk before the experiments were started.

The rats were anesthetized with 120 mg/kg body wt of 5-ethyl-5-(1-methylpropyl)-2-thiobutabarbitral sodium (Inactin, Sigma, St. Louis, MO), given intraperitoneally. The body temperature was maintained at 37–38°C, using a heating pad controlled by a rectal thermistor probe. To facilitate spontaneous breathing, a short PE-200 cannula was placed in the trachea. A PE-50 cannula containing heparin (Leo Pharma, Malmö, Sweden; 12.5 IU/ml) dissolved in 0.9% saline was placed in the right femoral artery to monitor blood pressure, and the right femoral vein was catheterized to enable a continuous infusion of Ringer’s solution (120 mM NaCl, 25 mM NaHCO3, 2.5 mM KCl, and 0.75 mM CaCl2) at a rate of 1 ml per h during the experiment. In some experiments the right femoral vein was also the route for administration of Nω-nitro-L-arginine (l-NNA, Sigma) or l-ω-N-(1-iminoethyl)-lysine (l-NIL, Sigma) in the Ringer’s solution. The colon was exteriorized through a midline abdominal incision, and the descending colon was opened longitudinally 1–2 cm. The rat was placed on its right side on a Lucite microscope stage. The colon was everted and loosely draped over a truncated cone. A double-bottom mucosal chamber with 37°C circulating water and a hole in the bottom was fitted over the mucosa, exposing ~0.5 cm² of the mucosal surface. The junction was sealed with silicon grease (Dow Corning high vacuum grease; Dow Corning, Weisbaden, Germany). The mucosal chamber was filled with ~5 ml 37°C saline to keep the tissue moist and warm. This model is further described by Atuma et al. (2, 13).

Colonic mucosal blood flow measurements. The colonic mucosal blood flow was measured continuously from the mucosal side by using laser Doppler flowmetry (PeriFlux 4001 Master and PeriFlux PF3; Perimed, Järfalla, Sweden). Red monochromatic laser light (wavelength 635 nm) is guided via an optic fiber down to the tissue. In the tissue the light is reflected against moving objects, and a shift (wavelength 635 nm) is guided via an optic fiber down to the tissue. The junction was sealed with silicon grease (Dow Corning high vacuum grease; Dow Corning, Weisbaden, Germany). The mucosal chamber was filled with ~5 ml 37°C saline to keep the tissue moist and warm. This model is further described by Atuma et al. (2, 13).

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After the preparation, the rats were given at least 1 h to stabilize before the blood flow measurements began. Systemic blood pressure and colonic blood flow had been at steady-state for at least 20 min before the experiment started. The rats were divided into four treatment groups: 1) saline intrarectally in a single dose 7 days prior to the experiment (TNBS control), 2) TNBS intrarectally in a single dose 7 days prior to the experiment, 3) untreated (DSS control), and 4) DSS given in the drinking water for 9 days.

The colonic mucosal blood flow was followed for 100 min in all four groups. In different sets of experiments, the NOS inhibitor l-NNA (10 mg/kg bolus in the volume of 1.0 ml followed by an infusion of 3 mg·kg⁻¹·h⁻¹ i.v with the infusion rate of 1.0 ml/h) or the iNOS inhibitor l-NIL (10 mg/kg bolus in the volume of 1.0 ml followed by an infusion of 3 mg·kg⁻¹·h⁻¹ i.v with the infusion rate of 1.0 ml/h) was given, starting 20 min after the start of the experiment, in all four groups. The recorded mean arterial blood pressure (expressed in mmHg) and mucosal blood flow (in PU) were used to calculate the vascular resistance during the experiments.

RNA purification. Another 12 rats (4 controls, 4 TNBS-treated, and 4 DSS-treated) were used for these experiments. The rats were anesthetized with 120 mg/kg body wt of 5-ethyl-5-(1-methylpropyl)-2-thiobutabarbitral sodium, and the distal colon was excited and immediately frozen in liquid nitrogen. To investigate changes in eNOS, iNOS, and nNOS, whole colonic tissue RNA was isolated using an RNA isolation kit (Peqlab, Erlangen, Germany). The tissue samples were homogenized in a mortar in liquid nitrogen. For the isolation of total RNA, 20 µg of homogenized tissue was used in accordance with manufacturer’s instructions.

mRNA quantification. The amounts of eNOS, iNOS, and nNOS mRNA were estimated by real-time RT-PCR analyses. Five hundred nanograms of the isolated RNA was reverse transcribed using SuperScriptIII Reverse Transcriptase and random hexamers (Invitrogen, Karlsruhe, Germany) in accordance with the manufacturer’s instructions. Quantitative PCR analysis was performed in triplicate
using a GeneAmp 5700 (Applied Biosystems, Darmstadt, Germany) as instructed by the manufacturer. SYBR Green was used for the fluorescent detection of DNA amplified during the PCR. The PCR reaction took place in a total volume of 25 \mu l with 0.2 pmol/\mu l of each primer, and SYBR Green master mix (Bioline, Luckenwalde, Germany). One microliter cDNA corresponding to 20 ng RNA was used as template. Published sequences for rat eNOS (NM_021838), rat nNOS (NM_052799), and rat \beta-actin (NM_031144) were used to design primers that bridge at least one intron. Primer sequences were as follows: eNOS sense 5′-CAG CAC CAG ACC ACA GCC CC-3′ and antisense 5′-TCC TGC TGA GCC TGT GCA CT-3′; iNOS sense 5′-CAG GTG CTA TTC CCA GCC CAA CA-3′ and antisense 5′-CAT TCT GTG CAG TCC CAG TGA GGA A-3′; nNOS sense 5′-ATC CAG GTG GAC AGA GAC CTC GAT G-3′ and antisense 5′-CCG AGG TAG GIG ACT GTT CCT TCT CT-3′; \beta-actin sense 5′-GTT CCA CAC CCG CCA CCA-3′ and antisense 5′-GCC ACC ACA GCC CC-3′; eNOS intron. Primer sequences were as follows: eNOS sense 5′-CCG AGG TAG GGG ACT GTT CCT TCT CT-3′; \beta-actin sense 5′-GTT CCA CAC CCG CCA CCA-3′ and antisense 5′-ACC CAT ACC CAC CAT CAC ACC CTG-3′. Conditions were 50°C, 2 min; 95°C, 10 min; 40 cycles: 95°C, 30; 67°C, 1 min; 77°C, 30 s. The levels of expression of the eNOS, iNOS, and nNOS mRNA were normalized to the levels of \beta-actin using the delta CT method. Parallelism of standard curves of the test and control was confirmed.

Chemicals. NaCl was obtained from Fluka Chemie, and NaHCO3, KCl, and CaCl2 from Merck, Buchs, Switzerland. NaCl (20 ml ampule, 9 mg/ml) was from Braun Medical.

Statistical analysis. All data are expressed as means ± SE. For statistical analysis two- and one-way ANOVA repeated measurements with Tukey’s multiple comparison test were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Student’s t-test was used for statistical evaluation of unpaired data. P values < 0.05 were considered significant.

RESULTS

Blood pressure and mucosal blood flow remained stable during the entire experimental period in all three groups, controls, TNBS-treated, and DSS-treated (Fig. 1A). Since there were no differences in the blood flow values between the untreated animals (group 1) and the animals given saline rectally 7 days prior to the experiments (group 3), these animals were regarded as one control group. When we compared the colonic mucosal blood flow, expressed in PU, during the control period (the first 20 min) in all experiments (n = 75), we found that it was significantly higher in both the TNBS-treated (184 ± 21 PU, n = 17) and the DSS-treated animals (189 ± 21 PU, n = 22) than in the controls (117 ± 7 PU, n = 36) (Fig. 2). When taking all experiments in account, there were no differences in mean arterial blood pressure during the control period between the groups (control, 100 ± 2 mmHg n = 36; DSS-treated, 97 ± 2 mmHg n = 22; TNBS-treated, 99 ± 4 mmHg n = 17).

NOS and Colonic Mucosal Blood Flow

L-NNA administered to control animals resulted in a significant rise in blood pressure (by 50%), but no significant changes in mucosal blood flow were observed. The calculated vascular resistance consequently increased (by 54 ± 11%), indicating that NO might be involved in maintaining baseline blood flow. Another theoretical possibility is that the blood flow is regulated by myogenic (or metabolic) response to the increased blood pressure. In TNBS- and DSS-treated rats a significant reduction in blood flow was seen during treatment with L-NNA (by 33 ± 4% and by 33 ± 7%, respectively) (Fig. 1B). As in the control animals, a significant rise in blood pressure was also seen in these rats when treated with L-NNA.
The calculated vascular resistance increased to a significantly greater extent in the TNBS- and DSS-treated rats (by 102 ± 10% and by 118 ± 10%, respectively) than in the control animals during L-NNA administration, indicating a higher level of vasodilating NO in colitis.

**iNOS and Colonic Mucosal Blood Flow**

Blood pressure and mucosal blood flow remained unchanged when L-NIL was given to control animals. Similarly, L-NIL administration did not affect either blood flow or blood pressure in the TNBS- and DSS-treated rats (Fig. 1C), suggesting that iNOS-induced NO was not involved in the blood flow increase in the rats with colitis. The used dose and route of administration of L-NIL has earlier been shown to inhibit iNOS in vivo (46).

**eNOS, iNOS, and nNOS mRNA Expression**

The relative expression of eNOS mRNA was significantly increased in the TNBS- and DSS-treated animals compared with controls (Fig. 3). iNOS expression was also significantly higher in the colitic animals compared with the controls. There was, however, no difference in nNOS expression between the groups (Fig. 3).

**Severity of Colitis**

**TNBS.** TNBS induced severe colitis with penetrating ulcers, a reduced colon length (control, 19.4 ± 0.4 cm; TNBS, 16.0 ± 0.4 cm), and thickening of the colonic wall (Fig. 4). All the animals in the TNBS-treated group had a damage score that was at least four times higher than the highest damage score in the control group. These colitic rats also had a higher colon weight per centimeter than the controls (TNBS, 0.16 ± 0.01 g/cm; controls, 0.09 ± 0.02 g/cm), and they lost body weight after the treatment with TNBS. The controls, on the other hand, gained weight.

**DSS.** DSS induced a more diffuse mucosal inflammation covering the entire colon and with a higher fecal blood content than in the TNBS-treated rats. In the DSS model, a shortening of the colon was observed (control, 19.4 ± 0.4 cm; DSS, 16.3 ± 1.1 cm), but no increase in thickness of the colonic wall or weight per centimeter was observed in these animals. The DSS-treated animals reached a DAI of 2.2 ± 0.1 at day 9 of DSS treatment, whereas none of the controls showed any signs of diarrhea, weight loss or bloody stool (Fig. 4).

**DISCUSSION**

In the present study we used two common models of experimental colitis, the TNBS and the DSS model. TNBS induced severe colitis with penetrating ulcers, a reduced colon length, and thickening of the colon wall (41). This model shares some of the histopathological features of Crohn’s disease (39). DSS is a model of ulcerative colitis, induced a more diffuse mucosal inflammation covering the entire colon, and resulted in diarrhea and gross rectal bleeding (5, 40, 43).

Colonic mucosal blood flow was measured by laser Doppler flowmetry, a well-established method for continuous measurements of mucosal blood flow (12, 25, 42). Our results show that irrespective of the disease model used, colitic rats have a higher colonic mucosal blood flow than control rats and that NO is involved in the regulation of the mucosal blood flow in the inflamed colon. Both iNOS and eNOS are upregulated in TNBS- as well as DSS-induced colitis, whereas nNOS remained unchanged. Inhibition of iNOS did not influence the colonic mucosal blood flow either in the control or in the colitic...
rats, whereas L-NNA, an unselective inhibitor of NOS, reduced the blood flow in the colitic rats, demonstrating the importance of constitutively expressed NOS in the blood flow increase seen during colitis.

Bacaner and Hultén et al. (3, 15) found an increased blood flow in inflamed regions of the bowel in patients with ulcerative colitis and Crohn’s disease. IBD comprises a group of inflammatory conditions with greatly heterogeneous signs and symptoms in the affected patients. Owing to this variation, it is very difficult to study these conditions in humans. Blood flow measurements in patients with IBD are difficult due to problems such as determination of the phase of the inflammation, limitations of the blood flow techniques, and the influence of the patient’s medication. It is therefore important to investigate the blood flow in animal models of colitis, where functional studies under controlled conditions can be performed.

Reports on the changes in mucosal blood flow in various experimental models of colitis provide conflicting findings. That acute inflammation involves hyperemia is not questioned, but in the chronic situation, both increased blood flow and reduced blood flow have been recorded.

Buhr and colleagues (7, 21–23), using intravital microscopy, have repeatedly observed an increased mucosal blood flow within 24 h after induction of colitis with TNBS and a reduced blood flow after 3 days. Deniz et al. (6) recorded decreased arterial mesenteric blood flow at early stages in TNBS-induced colitis in mice. The first days after TNBS administration, the induced colitis is still in an acute phase. In the present study, increased blood flow was noted 1 wk after TNBS administration, hence during a more manifest stage of the inflammation, which may account for the discrepancies seen. In accordance with our results, Hosoi et al. (14) found an increased mucosal blood flow (hydrogen gas clearance) 1 wk after induction of colitis with TNBS. The question whether the mucosal blood flow increases or decreases during TNBS-induced colitis might depend on where the measurements have been made. Most likely, there are regional differences in blood flow within the inflamed area, where deep penetrating ulcers are ischemic, whereas the surrounding mucosa is hyperemic. Our measurements were always performed about 2 cm proximal to the ulcerated area in the distal colon.

Inducing colitis with DSS renders a gradual disease progression, with a disease severity that increases with the time of DSS exposure. In the DSS model, a reduction in blood flow of small submucosal arterioles only was noted on days 4 and 6 in the mouse proximal colon (38). Another study, measuring blood flow in the proximal colon of mice, showed no blood flow changes during DSS treatment (52). However, a higher colonic blood flow was seen in the distal rat colon on days 6 and 7 with use of microspheres (8). This is in accordance with our findings of a higher colonic blood flow in the distal colon 9 days after the start of DSS administration. We and others (7, 9) have observed that the degree of inflammation in DSS-induced colitis tends to be greater in the distal than in the proximal colon, which might explain the differences in reported colonic blood flow.

Several studies have demonstrated that colitis is associated with changes in the colonic microvasculature. McLaren et al. (34) concluded that DSS-induced colitis in rats augments the capillary diameters and increases the vessel density in the colonic mucosa. Enlarged colonic vessels have also been found in biopsy samples from patients with Crohn’s disease and with ulcerative colitis (45). Recently Ravnic et al. (48) showed that the diameter of the colonic mucosal capillaries increased during TNBS-induced colitis in mice. They also measured the velocity of the blood flow in the mucosal plexus and found that it was decreased. Thus despite the decrease in velocity, due to the dilatation, the net effect was an increased blood flow to the mucosa. In our studies we noticed that the small mucosal vessels visible through the microscope were enlarged in both the DSS- and TNBS-induced colitis models, confirming the above described microvascular changes.

In the present study, a twofold upregulation of eNOS mRNA and a 20-fold upregulation of iNOS mRNA was detected both in DSS- and TNBS-induced colitis using real-time RT-PCR. nNOS mRNA was not significantly altered in either of the colitis models compared with the controls, which is in agreement with findings in a previous study of manifest DSS-induced colitis (4). In other studies, however, in which measurements were made only in the muscle layer of the colon, a reduced nNOS activity was observed in the DSS model, suggesting that this downregulation is responsible for impaired colonic peristalsis during colitis (19, 36). In line with this reduction, our study with measurement of the mRNA levels throughout the colonic wall, showed a slightly nonsignificant decrease in nNOS in the DSS-treated animals, which might have been significant if measurements had been conducted in the muscle layer alone.

The massive increase in iNOS mRNA we measure is not surprising, since iNOS is a hallmark of inflammation, and several earlier studies have demonstrated an upregulation of iNOS in mucosal samples both in DSS (4) and TNBS models (33), as well as in human patient data representing both Crohn’s disease and ulcerative colitis (45). iNOS has been localized in infiltrating macrophages and neutrophils in the colonic mucosa and submucosa, surrounding the crypts, and in epithelial cells both in animal models (35) and in patients with Crohn’s disease (58) and ulcerative colitis (45). The increase in iNOS in the tissue is correlated with the severity of the disease (57). The magnitude of increase in iNOS mRNA expression we detect during inflammation in our models indicates there may be production of a large amount of NO; however, specific inhibition of iNOS did not alter the blood flow or vascular resistance in our colitic rats. In addition, L-NNA, which is a poor inhibitor of iNOS but a potent eNOS and nNOS inhibitor (37), lead to a colonic blood flow reduction. These results are certainly surprising and difficult to explain. One possible explanation is that the NO produced by iNOS in the mucosa diffuses into the venules and is transported away from the arterioles in the submucosa without influencing the blood flow. eNOS, which is produced in the vessel wall, has a much greater influence of the vascular resistance than NO produced in mucosal cells.

The increase in eNOS mRNA observed in the present study during colitis confirms previous reports. Elevated levels of eNOS have been found both in colonic tissue from DSS-induced colitis in mice (4) and in mesenteric endothelial cells from patients with ulcerative colitis (16).

Recent results have indicated that eNOS might be involved in protecting the colon against mucosal inflammation, since the disease activity was increased in eNOS-deficient mice treated with DSS (51). Vallance et al. (56) confirmed these findings in
the TNBS model, where the eNOS-deficient animals suffered from more severe colitis. In the present study we have found indications that eNOS plays an important role in regulating the colonic mucosal blood flow during inflammation. When the NOS activity was blocked with the nonselective inhibitor, L-NNa [potency; eNOS = nNOS > iNOS (37)], the vascular resistance increased more in the colitic rats than in the controls, pointing to a higher level of vasodilating NO in these rats. Since eNOS, and not nNOS, is upregulated in the colitis models and since eNOS is expressed mainly in endothelial cells in close proximity to arteriolar smooth muscles, we can conclude that the blood flow alterations seen in the colitis models must probably depend on the eNOS activity.

In conclusion, we have shown in this study that mucosal blood flow is increased in the distal colon in both the TNBS- and DSS-induced colitis models. This blood flow increase is most probably due to upregulation of eNOS and not to the high iNOS levels recorded. One protective action of eNOS may therefore be to increase the mucosal blood flow during inflammation.

ACKNOWLEDGMENTS

We thank Annika Jägare and Ulrike Neumann for excellent technical assistance.

GRANTS

These studies were supported by grants from the Swedish Research Council (04X-08646).

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