Colonic blood flow responses in experimental colitis: time course and underlying mechanisms

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Inflammatory bowel diseases (IBD) in humans are associated with morphological alterations in the vasculature that appear to correspond with disease extent and severity. Dilated, tortuous vessels with luminal irregularities, increased vascularity, and congestion of mucosal and submucosal microvessels are characteristic vascular changes that occur during the early stages of gut inflammation (14, 19). However, reductions in vessel diameter, decreased vascular density, focal endothelial cell necrosis in venules, and hemorrhage are often observed in advanced stages of chronic gut inflammation with tissue remodeling. These vascular alterations suggest that gut blood flow is increased in the early phase but reduced in later stages of chronic inflammation. This conclusion is supported by studies on human subjects with ulcerative colitis, which reveal increased colonic blood flow during the early fulminant phase of inflammation, whereas a reduction in flow is detected in the chronically inflamed and remodeled colon (16). Most other studies in humans suggest that gut microvascular perfusion is significantly increased in IBD during periods of active disease (3, 11, 14, 19), and it has been reported that as much as 25% of cardiac output is diverted to the colon in patients with ulcerative colitis (compared with 4% normally) (16). The small number of studies in animal models of colitis have not revealed a consistent pattern of blood flow changes, with some models resulting in vasodilation and others vasoconstriction (4, 7, 21, 27). It remains unclear whether the more commonly employed animal models recapitulate the local and systemic (e.g., cardiac output) vascular responses to inflammation that have been described in human IBD.

The mechanism(s) that underlie blood flow responses to chronic intestinal inflammation remain poorly defined. However, the results of a recent study (13) provide novel insight into a mechanism that could explain the reduction in blood flow that is observed in the late remodeling stage of human IBD. Although arterioles from normal human intestinal submucosa were shown to dilate in a dose-dependent and endothelium-dependent manner to acetylcholine, similar-sized arterioles in the chronically inflamed intestine exhibited a significantly reduced dilation response to acetylcholine. A role for oxidative stress was proposed in the inflammation-induced impairment of endothelium-dependent vasodilation because the inflamed arterioles exhibited an enhanced oxidative stress response compared with control arterioles, and treatment with a superoxide dismutase (SOD) mimetic restored the acetylcholine-induced vasodilatory response in arterioles within the inflamed human intestine (13). These findings suggest that superoxide-dependent impairment of the dilatory capacity of arterioles may explain the reduction of tissue perfusion and oxygenation that has been detected in the chronically inflamed bowel. Although there are several potential sources [mitochondrial oxidases, xanthine oxidase, and NAD(P)H oxidase] of superoxide in the inflamed gut that could lead to the diminished bioavailability of nitric oxide (NO) and consequent impairment of arteriolar dilation, the source(s) has not been identified. Furthermore, it remains unclear whether a similar impairment of endothelium-dependent arteriolar dilation is also manifested in animal models of IBD.
The overall objective of the present study was to determine whether the changes in blood flow and arteriolar reactivity to endothelium-dependent vasodilators that have been described in the gut circulation (as well as cardiac output) during human IBD are recapitulated in a widely employed mouse model of experimental colitis: dextran sodium sulfate (DSS). Another major objective was to determine whether superoxide mediates an impaired endothelium-dependent vasodilatory response in colonic arterioles of mice with DSS colitis and to address the possible role of NAD(P)H oxidase as a source of superoxide in this model.

**MATERIALS AND METHODS**

*Mice and induction of colitis.* The animals used in the experiment were 6- to 8-wk-old male C57BL/6J mice [wild-type (WT) control strain] and hemizygous male NAD(P)H oxidase knockout (gp91phox-/-) mice obtained from Jackson Laboratory (Bar Harbor, ME). Heterozygous transgenic (TgN) mice [C57BL/6-Tg[NOD1]3Cje] carrying the Cu,Zn-SOD gene (SOD1) were bred in house and identified by qualitative demonstration of Cu,Zn-SOD using nondenaturing gel electrophoresis followed by nitroblue tetrazolium staining. All animals were housed under specific pathogen-free conditions in standard cages and were fed standard laboratory chow and water ad libidum until the desired age. Colitis was induced by the administration of 3% DSS (molecular weight, 40 kDa; ICN Biomedicals; Aurora, OH) in water ad libidum until the desired age. Colitis was induced by the administration of 3% DSS (molecular weight, 40 kDa; ICN Biomedicals; Aurora, OH) in water ad libidum until the desired age. Colitis was induced by the administration of 3% DSS (molecular weight, 40 kDa; ICN Biomedicals; Aurora, OH) in water ad libidum until the desired age. Colitis was induced by the administration of 3% DSS (molecular weight, 40 kDa; ICN Biomedicals; Aurora, OH) in water ad libidum until the desired age. Colitis was induced by the administration of 3% DSS (molecular weight, 40 kDa; ICN Biomedicals; Aurora, OH) in water ad libidum until the desired age.

**Assessment of inflammation in DSS-treated mice.** Daily clinical assessment of DSS-treated animals included measurement of drinking volume and body weight, evaluation of stool consistency, and the presence of blood in stools by a guiac paper test (Coloscreen, Helena Laboratories; Beaumont, TX) (5). A previously validated clinical disease activity index (DAI) (25) ranging from 0 to 4 was calculated using the following parameters: stool consistency, presence or absence of fecal blood, and weight loss.

**Echocardiographic assessment of cardiac function.** In vivo trans-thoracic echocardiography of the left ventricle (LV) using a 15-MHz linear array transducer (15L8) interfaced with a Sequoia C256 (Acuson; Mountain View, CA) was performed as described (18, 28). The animals were anesthetized with ketamine (150 mg/kg body wt) and xylazine (7.5 mg/kg body wt). Approximately 0.9 ml of blood was harvested via a catheter placed in the carotid artery. The blood was collected in polypropylene tubes containing 0.1 ml acid-citrate-dextrose buffer (Sigma-Aldrich; St. Louis, MO). RBCs were obtained by centrifugation (550 g for 10 min). The supernatant was removed, and the erythrocyte pellet was washed with PBS (pH 7.4) and centrifuged again at 550 g for 5 min. This step was repeated four to five times. After the supernatant was discarded, 100 μl of the RBC pellet were mixed with 1 ml PBS and incubated for 60 min at room temperature with 10 μl 1,1-didodecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiL; Molecular Probes, Eugene, OR; 50 mg/ml). The cells were then centrifuged at 550 g for 5 min, and the supernatant was discarded. The RBC pellet was washed four to five times as before. The fluorescently labeled RBC pellet was then resuspended in 1 ml PBS and protected from light until infusion into a recipient animal.

**Estimation of blood flow in colonic microvessels.** Fluorescently labeled RBCs were infused intravenously to measure RBC velocity in different-sized arterioles and venules of the colonic mucosa at days 0, 2, 4, and 6 of DSS colitis in C57BL/6J mice. The mice were anesthetized with ketamine (150 mg/kg body wt) and xylazine (7.5 mg/kg body wt) and placed on an adjustable acrylic microscope stage, and a laparotomy was performed. The animal was placed on its left side, the proximal (ascending) colon was exteriorized with moist cotton swabs, and the colon was covered with a nonwoven sponge and superfused at 37°C with bicarbonate-buffered saline (BBS) solution (pH 7.4). After infusion of the solution (50–150 μl) of labeled RBCs, microvessels were observed using a ×20 objective lens. Visualization of DiL (excitation: 549 nm, emission 565 nm) required a Chroma filter block with an excitation filter (535 ± 50 nm), a dichroic mirror (565 nm dichroic longpass), and a barrier filter (590 nm longpass).

**Vessel diameter was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX).** Single, unbranched vessels with diameters ranging 20–80 μm in arterioles and 20–100 μm in venules and a length of >150 μm were selected for study. In each animal, three to five vessels were examined randomly, and the results were calculated as the mean of each parameter in all vessels examined. The microscopic images were received by a charge-coupled device video camera (XC-77, Hamamatsu Photonics) that was attached to an intensifier (C2400, Hamamatsu Photonics), projected onto a monitor (PVM-2030, Sony), and recorded on a videocassette recorder (BR-S601MU, JVC) for off-line analysis. A video time-date generator (WJ810, Panasonic) displayed the stopwatch function onto the monitor. Blood flow in each vessel was calculated from the mean of product mean RBC velocity (Vmean) and microvessel cross-sectional area assuming cylindrical geometry, and wall shear rate (WSR) was calculated using the Newtonian definition: WSR = 8(Vmean/D), where D is vessel diameter.

**Endothelium-dependent arteriolar dilation.** Once the proximal (ascending) colon was exteriorized with moist cotton swabs, two incisions in the colonic wall were made with a radioknife (Geiger NY) to perfuse the exposed colon with BBS. The input port (P-190 polyethylene tube, Becton Dickinson) for luminal perfusion with BBS was near the junction of the cecum and ascending colon, whereas the output port was located ~10–15 mm downstream to avoid interference with vessel observation. The colonic segment was perfused with BBS (37°C, pH 7.4) using a circulator (Mulf pals-2, Gilson). The colonic segment under observation was covered with a nonwoven sponge soaked in warm BBS. The colonic microcirculation was observed with the inverted intravital microscope setup described above.

Single unbranched arterioles with diameters ranging between 15 and 50 μm were chosen for this series of experiments. The flow rate of the luminal perfusate BBS was set to 1 ml/min, and the animals were allowed to stabilize for 20–30 min. Rhodamine-6G (0.02%) was infused intravenously to visualize the microvasculature. After baseline measurements of diameter were obtained from each arteriole of...
interest, the endothelium-dependent vasodilator bradykinin (Sigma-Aldrich) was added to the colonic perfusate for 5–10 min, and arteriolar diameter was measured again in the same vessels. The perfusate was then replaced with BBS, and arteriolar diameter was allowed to return to baseline values. The concentrations of bradykinin used were 0, 10⁻⁴, 10⁻³, and 10⁻² mM dissolved in BBS (pH 7.4), progressing from the lowest to highest dose in each group of control and DSS-treated C57BL/6J mice, SOD TgN, and gp91(phox) knockout mice on day 6 of colitis. All preparations were then perfused with the endothelium-independent vasodilator papaverine (10⁻³ M) to determine whether the smooth muscle relaxation responses were comparable between groups, thereby allowing endothelial dysfunction to be distinguished from smooth muscle dysfunction. Arterioles that failed to respond to papaverine were not analyzed any further and excluded from the study. Arteriolar vasorelaxation responses to bradykinin and papaverine were expressed as a percentage of the baseline diameter.

**Statistics.** Standard statistical analyses, i.e., one-way ANOVA with Scheffe’s (post hoc) test, were applied to the data. All values are reported as means ± SE, with at least 5 mice/group. Statistical significance was set at $P < 0.05$.

**RESULTS**

**Body weight and disease activity index in WT mice.** Mice placed on DSS experienced a significant weight loss beginning on day 4, resulting in an 11% loss by day 6. The DAI followed a similar time course as previously reported (23), with a significant increase in DAI observed from day 4 (1.0 ± 0.1), which continued to increase through day 6 (2.9 ± 0.2).

**Cardiac function.** HR was significantly elevated in DSS-treated mice compared with their water-fed controls at day 6 (Table 1). Because the tachycardia was accompanied by reduced stroke volume in the DSS group, cardiac output and cardiac index did not differ between colitic and control mice. LV ejection fraction and %FS were similar between control and DSS-treated mice (data not shown).

**Blood flow in submucosal arterioles.** Blood flow was measured in a series of arterioles ranging in diameter from 20 to 80 μm in the submucosa of mice treated with DSS for 0, 2, 4, or 6 days. As expected, resting blood flow increased in accordance with arteriolar diameter (Fig. 1). In colitic mice, there was a significant reduction in arteriolar blood flow. This reduction in blood flow occurred in a time- and arteriolar diameter-dependent manner, with significant decrements in flow detected in the smallest arterioles (<25 and 25–39 μm) and only on days 4 and 6 of DSS colitis. An 18–30% reduction in blood flow was noted between day 0 and day 6 in arterioles with diameters ranging between 20 and 60 μm. Larger arterioles (60–79 μm) were marginally affected by the inflammatory response.

**Table 1. Cardiac output, cardiac index, heart rate, and stroke volume as measured by echocardiography in control mice and DSS-treated mice at day 6 of treatment**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac output, μl/min</td>
<td>12.2±0.89</td>
<td>10.8±1.31</td>
</tr>
<tr>
<td>Cardiac index, ml/min⁻¹·a body wt⁻¹</td>
<td>440.9±31.52</td>
<td>377.2±50.05</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>415.4±11.48</td>
<td>504.4±43.35*</td>
</tr>
<tr>
<td>Stroke volume, μl</td>
<td>30.0±2.88</td>
<td>21.3±1.32*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–10 mice/group. DSS, dextran-sodium-sulfate. *$P < 0.05$ vs. control.

The significantly diminished blood flow through the major resistance vessels (arterioles) in the inflamed colon also lead to an accompanying reduction in the shear rates generated by the movement of blood within different-sized postcapillary venules (Fig. 2). This reduction in shear rate, which ranged between 14% and 26%, was significant only on day 6 of DSS treatment. The largest reductions in venular shear rate were detected in the smallest venules.

**Arteriolar vasodilation responses in DSS colitis.** Arteriolar vasodilation responses (baseline diameters of 15–50 μm) were measured in the intact colon by perfusing the colon with different concentrations of the endothelium-dependent vasodi-
lator bradykinin. This caused a dose-dependent increase in arteriolar diameter with a 35% increase at 10^{-2} mM bradykinin compared with BBS alone (Fig. 3). In WT colitic mice, the vasodilation responses of colonic arterioles to bradykinin were significantly reduced (by >60%) compared with arterioles of control WT mice at any concentration of bradykinin. Responses of arterioles to the endothelium-independent vasodilator papaverine were slightly lower in colitic mice than in control mice, although this was not significant (Fig. 3, inset).

Role of NAD(P)H oxidase-derived superoxide in colitis-induced arteriolar dysfunction. TgN mice that overexpress the superoxide scavenger Cu,Zn-SOD were used to determine whether superoxide contributes to the impaired endothelium-dependent arteriolar dilation that accompanies DSS colitis. Although the SOD1 TgN mice did not exhibit any signs of protection against clinical indexes of colitis (20), the dilatory responses of colonic arterioles to bradykinin in the SOD TgN mice with DSS colitis were significantly improved, approaching the intensity observed in control WT mice (Fig. 3). Levels of vasodilation recorded in response to papaverine were comparable with both WT control and DSS-treated mice (Fig. 3, inset).

The role of NAD(P)H oxidase as a potential source of the superoxide that mediates the colitis-induced impairment of endothelium-dependent arteriolar dilation was assessed using gp91^phox/-/ mice. As previously reported, NAD(P)H oxidase deficiency did not alter the clinical progression of DSS colitis (20). However, the gp91^phox/-/ mice with DSS colitis did exhibit significantly improved arteriolar dilation in response to bradykinin compared with their WT DSS counterparts (Fig. 4). The improved endothelium-dependent arteriolar
dilation in colitic gp91^phox/-/ mice was nearly as dramatic as that observed in SOD TgN mice with DSS colitis, suggesting that NAD(P)H oxidase is the dominant source of superoxide that mediates the inflammation-induced impairment of arteriolar function in the colon. Similar to findings in SOD TgN mice, colitic gp91^phox/-/ mice exhibited endothelium-independent vasodilation responses to papaverine that were comparable with WT control and WT DSS groups (Fig. 4, inset).

**DISCUSSION**

The direction and magnitude of the blood flow changes that accompany chronic intestinal inflammation are likely to influence the quality and intensity of the inflammatory response as well as the ability of the intestine to recover from such a response. The tone of intestinal arterioles influences capillary hydrostatic pressure, a major determinant of capillary fluid filtration rate and the development of interstitial edema, which is a cardinal histopathological feature of gut inflammation (9). Inasmuch as arterioles represent the major site of resistance to blood flow in the gut microcirculation, the tone of these vessels also governs blood flow and the rate of oxygen delivery into the tissue. Because tissue perfusion and oxygenation are critical factors in wound healing, changes in arteriolar tone may influence the ability of the gut to heal ulcerated regions of the inflamed bowel (14). Similarly, arteriolar tone governs the flow of blood and shear forces that are generated in downstream postcapillary venules. Because shear forces are known to exert a significant influence on the ability of blood cells (leukocytes, platelets) to adhere in postcapillary venules (26), arteriolar tone can also influence the magnitude of the leukocyte infiltration that occurs during an inflammatory response.
Given that arteriolar function can impact on several elements of the inflammatory response, we chose to systematically analyze the changes in blood flow that occur in colonic arterioles during the development of DSS colitis. Our findings indicate that DSS colitis is associated with a time-dependent reduction in arteriolar blood flow that is confined to the smallest resistance vessels, i.e., arterioles with diameters <25 μm and between 25 and 39 μm. These relatively (18–30%) modest reductions in arteriolar blood flow were accompanied by comparable reductions in shear rate within downstream postcapillary venules. The peak changes in arteriolar flow and venular shear rate occurred during days 4–6 of DSS colitis, a time period that is known to correspond with intense inflammation and clinical signs of disease activity (23). Our finding that colonic mucosal blood flow is reduced in the later stages of DSS colitis is consistent with another intravital microscopic study of capillary blood flow changes during the acute stages of 2,4,6-trinitrobenzene sulfonic acid-induced colitis in rats, in which flow was significantly reduced, and the changes in capillary perfusion coincided with signs of acute inflammation and increased gut permeability (6). Although this model of DSS colitis is generally considered to resemble acute colitis in humans, the results obtained from the intravital microscopic analyses of blood perfusion in experimental colitis are generally consistent with the reduction in colonic blood flow that has been reported during the late remodeling stage of human ulcerative colitis (16). This is in contrast to reports from animal models of intestinal inflammation that describe an increased gut blood flow using different flow-measuring techniques (e.g., microspheres) (1, 7, 31), as has been found in the acute phase of human disease. For example, DSS treatment of BALB/c mice for 7 days was shown to result in an increased blood flow and decreased vascular resistance in the colon, particularly in the middle and distal portions (7). A definitive explanation for the discrepancy between the study in BALB/c mice and our own study (using C57Bl/6 mice) is not readily available; however, it may be related to mouse strain, DSS treatment regimen, and/or blood flow measurement techniques. The absence of an early increase in blood flow in our model that would be comparable to human disease may reflect differences in the initiating inflammatory stimulus or the accelerated time course of the disease in our model.

There are several possible explanations for the reduction in blood flow that we noted in colonic arterioles during the development of DSS colitis: 1) a systemic cardiovascular response that alters cardiac output and consequently results in an impaired perfusion of colonic arterioles; 2) a local vasomotor response that results from the release of vasoconstrictor agents (e.g., thromboxane); and 3) a diminished capacity of colonic arterioles to respond to endothelium-dependent vasodilators such as bradykinin or acetylcholine. The results obtained from our study address two of these possibilities. Although the colitic mice experienced tachycardia, possibly due to dehydration, anemia (30), and/or disease-associated pain, the observation that cardiac output/index did not differ between control and DSS-treated mice suggests that significant changes in cardiac function and systemic hemodynamics is an unlikely explanation for the blood flow responses noted in colonic arterioles.

Our findings related to the differential responses of colonic arterioles in control versus DSS-treated mice to the endothelium-dependent vasodilator bradykinin may have some bearing on the reduction of basal arteriolar blood flow seen in DSS colitis. Although the papaverine data suggest that decreased smooth muscle responsiveness may account for only a small portion of the colitis-induced decline in arteriolar function, it is likely that the endothelium plays a far more significant role in the impaired vasodilation observed in this model of inflammation. The impaired endothelium-dependent vasodilatory responses to bradykinin observed in DSS-treated mice are consistent with a recent report by Hatoum and co-workers (13), who showed that, whereas normal arterioles from human intestinal submucosa dilate in a dose-dependent and endothelium-dependent manner to acetylcholine, arterioles in the chronically inflamed intestine show a significantly reduced dilation response to acetylcholine. They suggested that this defective arteriolar response accounts for the reduced colonic blood perfusion that occurs during the late remodeling stage of human IBD.

Endothelium-dependent vasodilation has been demonstrated in virtually all vascular beds, and a defective response of arterioles to endothelium-dependent vasodilators has been described in many chronic pathologic conditions that include a significant inflammatory component such as atherosclerosis, diabetes, obesity, and hypertension (15, 17, 22, 29). NO plays a major role in mediating endothelium-dependent arteriolar dilation, and the inactivation of endothelial cell-derived NO by superoxide is frequently invoked to explain the impaired endothelium-dependent dilation that is associated with chronic inflammatory diseases (12, 24). There is evidence to support a role for superoxide in mediating the altered arteriolar responses observed in human IBD. Hatoum et al. (13) demonstrated that arterioles in the inflamed human colon exhibited an enhanced oxidative stress (compared with control arterioles) and that treatment with a SOD mimetic restored the acetylcholine-induced arteriolar dilation response within the inflamed human intestine. The findings of the present study indicate that the diminished responsiveness of arterioles to endothelium-dependent vasodilators that accompanies human IBD can be recapitulated in the DSS model of murine colitis and that the same mechanism may explain the defective dilatory responses of colonic arterioles in both human and murine IBD. Our observation that SOD1 transgenic mice with DSS colitis do not exhibit the impaired vasodilatory responses exhibited in WT colitic mice strongly supports a role for superoxide in this vasomotor dysfunction, probably as a result of its ability to inactivate NO.

Although the source of superoxide that mediates the defective colonic arteriolar responses in human IBD remains undefined, our studies on the arteriolar responses in murine DSS colitis strongly implicate the enzyme NAD(P)H oxidase. We found that colitic mice that were genetically deficient in gp91phox, a critical protein subunit of NAD(P)H oxidase, showed significant restoration of the arteriolar dilation response to bradykinin. The observation that colitic gp91phox−/− mice were not as effective in restoring the normal dilator responses to bradykinin as colitic SOD1-TgN mice suggests that NAD(P)H oxidase is not the sole source of the superoxide that mediates this response. The cellular source of NAD(P)H oxidase that generates the NO-inactivating superoxide in DSS colitis is not known, but endothelial cells and infiltrating leukocytes are likely possibilities. Although both cell types possess NAD(P)H oxidase with a functional gp91phox subunit (8, 10), neutrophilic NAD(P)H oxidase would be less likely to
generate superoxide in proximity to the source of NO (endothelium) because leukocyte-endothelial cell adhesive interactions in the inflamed colon are confined to postcapillary venules (23). However, extravasated neutrophils that migrate to positions adjacent to the abluminal surface of colonic arterioles may produce the superoxide that inactivates endothelial cell-derived NO. An alternative explanation may be that infiltrating leukocytes may release other inflammatory mediators that promote the generation of superoxide from the arteriolar endothelial cells, leading to dysfunction. This possibility is supported by the fact that blocking leukocyte-endothelial interactions in postcapillary venules attenuates impaired vasorelaxation responses after ischemia-reperfusion (2).

In conclusion, the results of this study indicate that blood flow in colonic arterioles is significantly reduced in a widely used model of experimental colitis. The decline in blood flow may result from the diminished capacity of arterioles in the inflamed colon to respond to endogenous endothelium-dependent vasodilators like bradykinin and acetylcholine. NAD(P)H oxidase-derived superoxide appears to play a major role in the induction of inflammation-induced endothelium-dependent arteriolar dysfunction. The reductions in arteriolar flow and venular shear rate may promote the inflammatory response through a shear rate-dependent recruitment of leukocytes and may delay tissue healing by rendering the colonic mucosa hypoxic. Additional studies are needed define the mechanisms that underlie the vascular defects that are associated with IBD and to better understand the pathophysiological consequences of inflammation-induced vascular responses.

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