Endothelium-dependent vasorelaxation plays a critical role in regulating tissue perfusion. Impaired vasorelaxation is a key feature of microvascular dysfunction that contributes to the pathophysiology of diabetes mellitus, hyperlipidemia, and atherosclerosis. A new association of microvascular dysfunction with clinical inflammatory bowel disease (IBD) (Crohn disease, ulcerative colitis) has been made. IBD is characterized by refractory, poorly healing mucosal ulcerations, and impaired endothelium-mediated vasorelaxation. Using in vitro dimension measurements of cannulated 50 to 200 μm arterioles, we have demonstrated that normal intestinal microvessels dilate in response to the classic endothelium-dependent agonist, acetylcholine (Ach). After inhibiting nitric oxide (NO) synthase, cyclooxygenase (COX), and hyperpolarization mechanisms, or after endothelial denudation, constriction was observed. Microvessels isolated from chronically inflamed IBD tissue demonstrated loss of NO-mediated and hyperpolarization-mediated vasodilation in response to Ach. However, in contrast to vessels from subjects without IBD or from uninvolved segments of bowel from patients with IBD, arterioles from active IBD segments had attenuated dilation to Ach that was independent of NO or hyperpolarization mechanisms. This dilation was eliminated by inhibiting cyclooxygenase, suggesting a prostanoid etiology.

The increased reliance on prostanoids in IBD to maintain vasorelaxation prompted investigation of the presumed endothelial source of prostanoids that was recruited to compensate for the loss of endothelial derived NO and endothelial-derived hyperpolarizing factor (EDHF). Herein we report an unexpected and novel mechanism of dilation that occurs in human intestinal arterioles from chronically inflamed IBD, which involves impaired endothelium-dependent release of a PG from nonendothelial sources.

**Materials and Methods**

The Medical College of Wisconsin’s Institutional Review Board approved all protocols. Human submucosal arterioles were dissected from full-thickness intestinal specimens obtained from patients undergoing clinically indicated bowel operations. Demographic data and diagnoses were obtained from hospital records and recorded at the time of surgery. After resection, preservation of tissue samples was performed as previously reported.
**Materials**

Endothelin-1 (Peninsula Laboratories, Inc, San Carlos, Calif) was prepared in saline with 1% bovine serum albumin, and used in concentrations from $10^{-10}$ to $10^{-7}$ mol to achieve the designated 30% to 50% vasoconstriction. All reagents were obtained from Sigma Company (St. Louis, Mo). INDO was dissolved in saline with NaCO₃. N5398 was supplied by Cayman (Ann Arbor, Mich). (3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino) hydantoin (BWA868C; Sigma Aldrich) and manganese (II) tetra (4-benzoic acid) porphyrin chloride (MnTBAP) were dissolved in ethanol. Other agents were prepared in distilled water. Final molar concentrations of agents in the organ chambers are reported. The addition of pharmacological agents produced <1% change in the volume of the circulating bath. Pharmacological antagonists did not produce significant changes in baseline microvessel diameter.

**Statistical Analysis**

Percent dilation was calculated as the percent change from the constricted diameter to the maximal passive diameter (at 60 mm Hg pressure), which was generally the diameter after papaverine (10⁻⁵ mol). Percent constriction was determined by calculating the percent reduction from maximal diameter after the application of ET-1. Comparisons of percent vasodilation under different treatments were performed using a two-factor repeated measures ANOVA using SAS proc mixed modules with auto-regressive covariance assumptions. Both computations were followed by a Bonferroni correction when significant differences were noted. To compare the sensitivities of the agents used, ED₅₀ values (negative logarithm of the molar concentration of a vasodilator that produced 50% of the maximal dilation to the agonist) were calculated. Maximal percent dilations and ED₅₀ values were compared by Student paired t test. Statistical significance was defined as $P<0.05$. All data are described as mean±SEM. $n$ indicates the number of microvessels. Only one vessel was used from each subject for any specific protocol.

**Results**

**Microvessel Characteristics**

Submucosal microvessels from 34 patients (25 with IBD and 9 without IBD [controls]) were used. The average resting diameter was 146.9±23.7 μm at 60 mm Hg pressure. All IBD specimens were from grossly actively inflamed areas of bowel. Patient demographics, the number of vessels used for each protocol, mean luminal diameter, gender, and surgical procedures are summarized in the Table.

**Impaired Endothelium-Dependent Dilation in IBD**

Vessels isolated from histologically normal non-IBD control bowel, demonstrated a prominent dose-dependent dilation to Ach (maximal diameter [MD]: 89±9%; Figure 1) similar to our previous report. After endothelial denudation of non-IBD control vessels, Ach-induced vasodilation was converted to vasoconstriction. Vessels from subjects with IBD also dilated to Ach but to a substantially less degree than controls. In contrast to controls, denudation did not affect the dilation in IBD (Figure 1).

Because the endothelial denudation procedure did not influence the Ach-induced dilation of IBD microvessels, we...
examined endothelial integrity in denuded microvessels using Dil-ac-LDL uptake and fluorescence microscopy. Uptake of DiI-ac-LDL and tissue fluorescence was similar in intact non-IBD and in IBD diseased segments (290 ± 10 U versus 300 ± 14 U in diseased segments, n=2, P=NS). Endothelial denudation resulted in a significant reduction in fluorescence intensity compared with intact vessels in IBD and control (290 ± 10 U versus 145 ± 2%, n=2; 300 ± 14 U versus 137 ± 6%, n=2). This finding was also evident when comparing CD-31 immunohistolabeled vessels, revealing absence of CD-31, an endothelial-specific protein, only in denuded vessels (data not shown). These studies demonstrate that the method of denudation used was effective in mechanically removing the endothelium in IBD arterioles and indicate the dilation to Ach in IBD involves a nonendothelial mechanism.

The Role of Reactive Oxygen Species in Endothelium-Independent Vasodilation in IBD

When compared with uninvolved IBD and control microvessels, chronically inflamed IBD microvessels demonstrated high levels of reactive oxygen species, including superoxide. To determine whether the excess vascular superoxide found in IBD contributes to diminished endothelium-independent dilation, experiments were conducted with the cell-permeable superoxide dismutase mimic, MnTBAP. IBD denuded microvessels pretreated with MnTBAP (10⁻⁴ mol; 60-minute incubation), demonstrated similar Ach-induced vasodilation compared with denuded vessels treated with vehicle (20±3% to 23±2%, respectively; Figure 2).

Role of Prostaglandins

Figure 3A shows that nonspecific inhibition of cyclooxygenase with INDO completely eliminates Ach-induced vasodilation in denuded IBD microvessels, with conversion to a frank constriction (15±2% to −32±7%, respectively). This contrasts with the lack of effect of INDO on Ach-induced

Demographics and Characteristics of Patients and Arterioles

<table>
<thead>
<tr>
<th>Type of Tissue</th>
<th>UC</th>
<th>CD</th>
<th>Non-IBD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, n=34</td>
<td>41 (30–60)</td>
<td>33 (22–55)</td>
<td>58 (40–76)</td>
<td>43 (22–76)</td>
</tr>
<tr>
<td>Male/female</td>
<td>6/5</td>
<td>10/4</td>
<td>6/3</td>
<td>22/12</td>
</tr>
<tr>
<td>N of vessels</td>
<td>24</td>
<td>54</td>
<td>48</td>
<td>126</td>
</tr>
<tr>
<td>% Dilation of intact vessels to Ach (10⁻⁴ mol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16±2#</td>
<td>15±5#</td>
<td>89±9</td>
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</tr>
<tr>
<td>Female</td>
<td>15±2</td>
<td>13±4</td>
<td>87±10</td>
<td></td>
</tr>
<tr>
<td>% Dilation of denuded vessels to Ach (10⁻⁴ mol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>11±2#</td>
<td>8±3#</td>
<td>−34±10*</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10±2</td>
<td>8±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal diameter (µmol)±SEM</td>
<td>156±11</td>
<td>145±13</td>
<td>140±15</td>
<td></td>
</tr>
</tbody>
</table>

Surgical procedures

- Small bowel resection: 0 12 2 14
- Large bowel resection: 11 2 2 15
- Bowel tumor resection: 0 0 5 5

**Patient demographics, diameter change in response to Ach (10⁻⁴ mol).**
Positive N represents dilation and negative N represents constriction.

All vessels were initially constricted with ET-1 to achieve a 30% to 50% reduction in maximal diameter) in intact and denuded vessels, mean luminal diameter, N of vessels, gender, and surgical procedure, grouped by experimental protocol and by condition (non-IBD control, and IBD [UC vs CD]). Percent dilation was calculated as the percent change from the constricted diameter to the maximal passive diameter. The luminal diameter is maximal passive diameter at 60 mm Hg pressure. Data shown as mean±SD.

#P<0.05 vs non-IBD (intact and denuded).
*P<0.05 vs intact non-IBD.
constriction of denuded microvessels from subjects without IBD (−34±10% and −37±12%, respectively, *P=NS; Figure 3B). To further investigate the specific cyclooxygenase subtype involved, dilation to Ach was determined in the presence of NS398 (1 μmol/L), a selective COX2 inhibitor. NS398 significantly inhibited vasodilation to Ach (21±1% to 38±9%; Figure 3C), eliciting a frank constriction similar to that produced by INDO. These data suggest that prostaglandins originating from nonendothelial cells play a key role in preserving Ach-induced dilation of IBD arterioles, but appear to play no role in nondiseased arterioles.

**Effect of Ach on Arachidonic Acid Metabolism**

Endothelium-denuded arterioles were incubated with 14C-arachidonic acid with or without Ach (10 μmol/L). PG metabolites were not detected in unstimulated vessels (Figure 4A). Ach did not elicit production of PG metabolites in arteries from patients without IBD. However, in arteries from patients with IBD, Ach stimulated production of a metabolite that comigrated on HPLC with PGD2, in an INDO-inhibitable manner (Figure 4B).

**PG Release and the Effect of a PGD2 Receptor Antagonist on Dilation to Ach**

Figure 5A shows that PGD2 produced a concentration-dependent vasodilation (66%±4%, *n=6) in human IBD mucosal arterioles. To investigate the role of PGD2 receptors in the vasodilation to Ach, mucosal arterioles were examined in the presence of BWA868C (1 μmol/L), a PGD2 receptor antagonist. BWA868C reduced dilation to PGD2 (66%±4% versus 19%±4%; *P<0.05; Figure 5A) and elicited a constriction to Ach in denuded vessels similar to that produced by INDO (3%±6% versus −33%±5%; *P<0.05; Figure 5B). These results are consistent with a role for PGD2 in the mucosal arteriolar dilation to Ach in patients with IBD.

**Discussion**

The major finding of this study is the identification of a novel mechanism of vasodilation in the human gut. In arterioles from patients with IBD, dilation to Ach, albeit diminished, is dependent on nonendothelial production of prostaglandins. This dilation is likely mediated by PGD2 released from the arteriolar media or adventitia and maintains ~60% of the dilation to Ach observed in vessels from subjects without IBD. However, in non-IBD subjects, the entirety of dilation to Ach arises from substances released from the endothelium. To our knowledge, this is the first demonstration of a shift in the mechanism of dilation from the endothelium to the underlying vascular tissue in response to chronic disease. This compensatory dilation was demonstrated both pharmacologically and analytically using HPLC.

The gastrointestinal system has its own intrinsic set of nerves known as the internal plexus or intestinal enteric nervous system, located in the walls of the gut. The mesenteric neurovasoregulation appears to involve three types of intestinal nerves and their respective transmitters. The sympathetic vasomotor nerves mediate constrictor responses via...
their transmitters, namely, norepinephrine, ATP, and neuropeptide Y. The parasympathetic nerves elicit vasodilation via release of acetylcholine and vasoactive intestinal peptide. The nonadrenergic noncholinergic vasodilator nerves release calcitonin gene-related peptide, substance P, and ATP. Accordingly, neurotransmitter-evoked intestinal vasodilation is mediated mainly by peptidergic and cholinergic receptors. In addition, to direct smooth muscle vasodilator effects of neurotransmitters released by parasympathetic and NANC nerves, there are also putative indirect smooth muscle relaxing effects involving neurotransmitter stimulation of the endothelium and mast cell release of vasodilator mediators.

Prostaglandins are small lipid molecular derivatives of arachidonic acid that regulate numerous cellular and tissue processes including vasomotor tone, platelet aggregation, neurotransmitter release, and immune function. In addition, prostaglandins play a key role in many aspects of gastrointestinal homeostasis and mucosal defense. The central importance of prostaglandins in epithelial mucosal function has been demonstrated by the ulcerogenic effect of nonsteroidal antiinflammatory drugs (NSAIDs) in the gastrointestinal tract. This is consistent with the protective effect of prostaglandins against ulcers in the gastrointestinal tract of both human and animal models.

Elevated concentrations of prostaglandins in IBD were first described by Gould et al, and confirmed by others. NSAIDS (nonspecific PG inhibitors) have been found to exacerbate both human IBD and animal models of chronic gut inflammation. Our observation of enhanced production of PGD2 via COX2 in IBD is consistent with prior literature (see Results). Wallace et al showed that normal colon expresses low levels of COX. Furthermore, in colitis, tissues markedly upregulate COX2 expression relative to COX1. The resulting increase in PGD2 production mediated downregulation of neutrophil infiltration into the mucosa, possibly contributing to reduction in disease acuity. Our findings of enhanced PGD2 production and preserved dilation to Ach suggest that COX2 mediated production of PGD2 also helps to preserve tissue perfusion in the inflamed intestine.

Traditionally, in disease states such as diabetes mellitus and hyperlipidemia in which NO is reduced, EDHF and/or prostaglandins are invoked as the mediators of vasodilation. Lamping et al showed that in coronary arteries from wild-type mice, dilation to Ach is mediated primarily by NO, whereas in endothelial NO synthase knockout mice, the dilation is dependent on activity of nNOS and COX. Sun et al showed that flow-induced dilation is mediated by both endothelial NO and prostaglandins in skeletal muscle arterioles from wild-type mice, but is mediated exclusively by prostaglandins in male endothelial NO synthase knockout mice. In each case the compensatory PG dilator was derived from the endothelium. These data suggest that certain vessels have the ability to compensate for the loss of one endothelium-dependent vasodilator mechanism by upregulating an alternative endothelium-dependent dilator system (EDHF or prostaglandins).

The present study demonstrates a novel compensatory mechanism, which does not require the endothelium, yet is dependent on COX to preserve dilation to Ach. Such compensation points to the importance of endogenous vasodilator mechanisms and/or PG production to preserve blood flow in the gut mucosa. These findings may also help explain the toxicity of NSAIDS in the treatment of IBD where their use results in the impairment of the sole remaining microvascular dilator mechanism to Ach, and presumably parasympathetic activation in the mucosa.
Study Limitations
Several potential experimental limitations should be considered. We examined only one endothelium-dependent dilator, Ach. It is possible that dilation to other endothelium-dependent agonists might not use a similar compensatory mechanism. One possible explanation for the failure of Ach to constrict denuded IBD mucosal arterioles is that muscarinic receptors are not present, inactivated, or internalized in vascular smooth muscle cells and are trafficked to the surface only in control. Another explanation is that PGD₂ is not produced in non-IBD vessels, as demonstrated in the HPLC data. However, in IBD the production of PGD₂ may overcome the effect of Ach on muscarinic receptors leading to dilation. Regardless of the mechanism, we are unaware of an antecedent for this type of compensatory dilation.

Because the size of the arterioles we used was quite small, global analyses including HPLC were used and localization of specific cellular sources of PGD₂ (ie, vascular smooth muscle, fibroblasts, mast cells, or inflammatory cells) was not possible. Future studies will be needed to determine the cell type responsible.

The small sample size for HPLC experiments was caused by the difficulty in obtaining sufficient mass of vascular tissue from the diseased bowels required for the incubations. Despite this difficulty, small variability between samples was observed, and the metabolite identified that comigrated with PGD₂ was only observed from inflamed bowel arterial incubation.

All vessels used in these studies were derived from surgical specimens and therefore represent a heterogeneous collection of tissue (ie, different degrees of active inflammation, various stages of disease, diverse patient ages, treatments, surgical duration, and anesthesia). Because all patients underwent surgery for symptomatic disease, it is likely that our patient population represents more advanced and severe disease. We were not able to control many of these potentially confounding factors, but for several reasons we believe that our approach is valid. First, we performed paired comparisons between vessels from the same patient, one treated with vehicle, the other with antagonist. This helped to limit the confounding influence of external factors such as medications, age, gender, and severity of disease. Second, we performed statistical analyses to examine the effects of age, gender, and disease (CD versus UC) on vasomotor responses. None of these conditions appeared to influence vasomotor responses. Third, vessels were superfused with substantial amounts of buffer before study (dissection of the vessel in PSS, incubation, and superfusion with fresh PSS for 1 hour before study), which likely removed most residual pharmacological agents from the tissue.

Despite this limitation, we believe that the difficulties associated with the inability to control confounding variables in clinical studies are offset by the relevance of studying responses in human tissue especially during chronic disease processes that may not be adequately mimicked in animal models.

Finally, our premise of a subendothelial compensatory dilator response depends on the adequacy of endothelial removal by air injection. This technique has been validated in intact arterioles from a variety of tissues, but not in IBD mucosal arterioles. Therefore, we also performed direct histological studies of endothelial integrity which demonstrated absence of the endothelial layer. This together with the absence of Ach-induced dilation and preserved dilation to papaverine in air-treated control vessels indicates that this denudation technique is suitable for vessels from subjects with IBD.

Summary
We have characterized a novel compensatory mechanism of vasodilation, which emerges in the setting of microvascular dysfunction in chronic human gut inflammation. In this condition, Ach produces a modest vasodilation, which is the net result of both an endothelium-independent vasodilation and direct vasoconstriction in mucosal arterioles. When the endothelium is removed, the dilation is unaltered, being maintained by vasodilator prostanooids arising from underlying tissue in response to Ach. The mediator is likely PGD₂, which is released from IBD vessels treated with Ach, but not from similarly treated normal intestinal arterioles devoid of endothelium.

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References


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Ossama A. Hatoum, Kathryn M. Gauthier, David G. Binion, Hiroto Miura, Gordon Telford, Mary F. Otterson, William B. Campbell and David D. Gutterman

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