Novel Mechanism of Vasodilation in Inflammatory Bowel Disease

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Objective—Endothelium-dependent dilation to acetylcholine (Ach) is reduced in mucosal arterioles from patients with inflammatory bowel disease (IBD). The contributions of both nitric oxide (NO) and endothelial-derived hyperpolarizing factor (EDHF) are decreased. We hypothesized that the remaining dilation results from products of cyclooxygenase.

Methods and Results—High-performance liquid chromatography (HPLC) was used to isolate eicosanoid vasodilator products and videomicroscopy was used to examine vasomotor responses in human mucosal arterioles from subjects with or without IBD undergoing bowel resection surgeries. In subjects without IBD, Ach constricted (−52% ± 10%) arterioles devoid of endothelium. Indomethacin (INDO) (cyclooxygenase inhibitor) had no effect. In contrast, Ach dose-dependently dilated both intact and endothelial denuded arterioles from patients with IBD. The dilation was converted to constriction by INDO (−54% ± 9%; P < 0.05 versus non-IBD) or by BWA868C (PGD2 receptor antagonist). Only in arterioles from subjects with IBD did Ach produce an arachidonic acid metabolite that comigrated on HPLC with PG D2 (PGD2). Exogenous PGD2 dilated (max = 66% ± 4%) IBD arterioles.

Conclusion—in arterioles from IBD patients, Ach-mediated dilation shifts from endothelial production of NO and EDHF to nonendothelial generation of a PG, likely PGD2. This is a novel dilator mechanism arising from nonendothelial vascular tissue that compensates for loss of endothelium-dependent dilation. PGD2 appears to be important in regulating mucosal blood flow in patients with IBD, implicating potentially detrimental effects from nonsteroidal antiinflammatory drugs. (Arterioscler Thromb Vasc Biol. 2005;25:2355-2361.)

Key Words: cyclooxygenase ■ inflammatory bowel disease ■ microcirculation ■ prostaglandin ■ vasodilation

Endothelium-dependent vasorelaxation plays a critical role in regulating tissue perfusion.1 Improved vasorelaxation is a key feature of microvascular dysfunction that contributes to the pathophysiology of diabetes mellitus, hyperlipidemia, and atherosclerosis.2,3 A new association of hyperlipidemia, and atherosclerosis.2,3 A new association of hyperlipidemia, and atherosclerosis.2,3 A new association of hyperlipidemia, and atherosclerosis.2,3 A new association of...
**Video microscopy**

Video microscopy was performed as previously reported. Briefly, isolated microvessels (50 to 200 μm in diameter) were transferred to a 20-mL organ chamber containing Krebs solution of the following composition (in mM/L): NaCl 118, KCl 4.7, CaCl2 2.5, KH2PO4 1.2, MgSO4 1.2, NaHCO3 20, Na2EDTA 0.026, and glucose 11, pH 7.4. Each end of the arteriole was secured to a separate glass micropipette (25- to 50-μm internal diameter) filled with Krebs buffer and transferred to the stage of an inverted microscope (CK2; Olympus) coupled to a charge-coupled device camera (WV-BL200, Panasonic) and video micrometer (VIA-100K; Boeckeler Instruments, Inc). Internal vascular diameters were measured throughout the experiment with a manually adjusted video-caliper and vessels were pressurized to 60 mm Hg using a hydrostatic reservoir. The chamber solution was continuously re-circulated at 30 mL/min, aerated with 20% O2, 5% CO2, and 75% N2, and maintained at 37°C by an external heat exchanger. All pharmacological agents were added to the external bath solution.

**Experimental Protocols**

After 60 minutes of equilibration, endothelin-1 (10−10 to 10−8 mol/L) was added extraluminally to constrict the vessels to a final diameter of 50% to 70% of the passive diameter. Ach (10−9 to 10−7 M) was added to the circulating bath solution in cumulative fashion, and steady-state diameters were recorded after each concentration (3 to 5 minutes). At the end of each experiment, maximal diameter was determined by adding papaverine (10−5 mol/L). The chamber then was washed with 500 mL of fresh buffer over 20 to 30 minutes. Inhibitor(s) (or vehicle) were added to the circulating bath for a second 30-minute equilibration, followed by endothelin-1 constriction and a second concentration–response curve. In some cases, experiments were repeated after endothelial denudation with air infusion, as described below.

**Endothelial Denudation, Morphological Assessment, and Stimulation**

In some vessels, the endothelium was mechanically denuded. Approximately 2 mL of warmed air was infused through the vessel over a 2-minute period. This was immediately followed by warmed physiological saline solution to fill the vessel and remove all air bubbles. The arterioles were pressurized and stabilized for 30 minutes before proceeding. This approach results in functional denudation of isolated arterioles without damage to the vascular smooth muscle. Studies of denuded vessels were run parallel to denudation of isolated arterioles without damage to the vascular smooth muscle. Histological assessment of endothelial integrity was also determined using acetylated low-density lipoprotein labeled with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (Di-ac-LDL) uptake and fluorescence microscopy. In the microvascular endothelium a functional scavenger receptor takes up the fluorescently tagged modified human low-density lipoprotein, DiI-LDL, labeled with 1,1-dioctadecyl-3,3,3,3-tetramethyl-indocarbocyanine perchlorate (Biomedical Technology, Inc, Stoughton, Mass) as demonstrated by fluorescence microscopy, indicating presence of viable endothelial cells.

**Metabolism of [14C] Arachidonic Acid**

Endothelium-denuded submucosal intestinal arteries (3 to 5 each) were incubated in 1 mL of HEPES buffer at 37°C with 0.5 μCi (10−9 mol/L) [14C] arachidonic acid (AA) (1 μCi/mmol; New England Nuclear, Boston, Mass) for 15 minutes. In some instances the arteries were pretreated with vehicle (1:1000 dilution of 95% ethanol) or indomethacin (10−5 M) for 10 minutes. Ach (10−7 M) was added and incubations were continued for an additional 10 minutes. The reactions were stopped by the addition of 150 μL 5% trichloroacetic acid (10 μL) to acidify the media to pH <3.5. The organic phase was extracted with 50% cyclohexane in ethyl acetate. The organic phase was collected, dried under a stream of nitrogen, and stored at −40°C. The sample was subjected to reverse-phase HPLC with a Nucleosil C8 column (5 mm, 4.6×250 mm; Phenomenex, Torrence, Calif) and a Beckman liquid chromatograph (model 112; Beckman Instruments, Fullerton, Calif) as previously described. Solvent A was 0.025 mol/L phosphoric acid in distilled water and solvent B was acetonitrile. An isotropic solution (40 minutes) was used with 31% solvent B in solvent A at a flow rate of 1 mL/min followed by a 35-minute linear ramp to 100% solvent B. Column eluates were collected at 2 fractions/min and radioactivity was analyzed by liquid scintillation spectrometry.

**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 10−8 mol/L, to achieve the designated 30% to 50% vasoconstriction. All reagents were obtained from Sigma Company (St. Louis, Mo). INDO was dissolved in saline with Na2CO3. NS398 was supplied by Cayman (Ann Arbor, Mich). (3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethyl)-amino) hydantoin (BWA868C; Sigma Aldrich) and manganese (III) 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 (10−4 mol/L). 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, ED90 values (negative logarithm of the molar concentration of vasodilator that produced 50% of the maximal dilation to the agonist) were calculated. Maximal percent dilations and ED90 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 ± 14; 300 ± 14 U versus 137 ± 6, respectively; 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.4 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 mimetic, MnTBAP. IBD denuded microvessels pretreated with MnTBAP (10^{-4} mol/L; 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 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 \text{\mu mol/L}), a selective COX2 inhibitor. NS398 significantly inhibited vasodilation to Ach (21\% \pm 1\% to 38\% \pm 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 $^{14}$C-arachidonic acid with or without Ach (10 \text{\mu mol/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 PGD$_2$, in an INDO-inhibitable manner (Figure 4B).

**PG Release and the Effect of a PGD$_2$ Receptor Antagonist on Dilation to Ach**

Figure 5A shows that PGD$_2$ produced a concentration-dependent vasodilation (66\% \pm 4\%, n=6) in human IBD mucosal arterioles. To investigate the role of PGD$_2$ receptors in the vasodilation to Ach, mucosal arterioles were examined in the presence of BWA868C (1 \text{\mu mol/L}), a PGD$_2$ receptor antagonist. BWA868C reduced dilation to PGD$_2$ (66\% \pm 4\% versus 19\% \pm 4\%; P<0.05; Figure 5A) and elicited a constriction to Ach in denuded vessels similar to that produced by INDO (3\% \pm 6\% versus −33\% \pm 5\%; P<0.05; Figure 5B). These results are consistent with a role for PGD$_2$ 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 PGD$_2$ released from the arteriolar media or adventitia and maintains \approx 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. Ac-
cordingly, 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 inhibit PGD{	extsubscript{2}} production in IBD, suggesting a role for these compounds in the pathogenesis of IBD.

**Figure 4.** Effect of Ach on \( ^{14} \text{C}-\)arachidonic acid metabolism. A. Arteries were incubated with or without Ach (10 \( \mu \text{mol/L} \)) and metabolites were extracted from the media and resolved by reverse-phase HPLC. The migrations of known PG standards are shown with arrows above each graph. Ach stimulates the production of a nonendothelial cell-derived arachidonic acid metabolite that comigrates with PGD{	extsubscript{2}} (n=3). Notice the differences in the scale between control and IBD results. B. Ach-stimulated production of PGD{	extsubscript{2}} was abolished after incubation with INDO.

**Figure 5.** Effect of PGD{	extsubscript{2}} receptor blockade on dilation of mucosal gut arterioles to PGD{	extsubscript{2}} and Ach. A. PGD{	extsubscript{2}} induces dilation in a concentration-dependent manner (n=6). This dilation was inhibited in a dose-dependent manner to BWA868C. B, Vasodilation to Ach was replaced with constriction in the presence of BWA868C, similar to INDO (n=5). \( P<0.05 \) vs IBD and/or IBD-denuded. Values represent mean±SEM.
exacerbate both human IBD and animal models of chronic gut inflammation.26

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.27 Furthermore, in colitis, tissues markedly upregulate COX2 expression relative to COX1.27 The resulting increase in PGD2 production mediated downregulation of neutrophil infiltration into the mucosa, possibly contributing to reduction in disease acuity.28,29 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.9,30 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 neuronal nitric oxide synthase and COX.31 Sun et al showed that flow-induced dilation is mediated by both endothelial NO and prostaglandins in skeletal muscle arteries from wild-type mice, but is mediated exclusively by prostaglandins in male endothelial NO synthase knockout mice.32 In each case the compensatory PG dilator was derived from the endothelium.31,32 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 exacerbate both human IBD and animal models of chronic gut inflammation.26 These mechanisms may not be adequately mimicked in animal models.

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 a precedent 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 incubations. 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,33,34 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 underly-
ing 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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