Increased arginase activity and endothelial dysfunction in human inflammatory bowel disease

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Increased arginase activity and endothelial dysfunction in human inflammatory bowel disease. Am J Physiol Gastrointest Liver Physiol 292: G1323–G1336, 2007. First published January 11, 2007; doi:10.1152/ajpgi.00499.2006.—Nitric oxide (NO) generation from conversion of L-arginine to citrulline by nitric oxide synthase isofoms plays a critical role in vascular homeostasis. Loss of NO is linked to vascular pathophysiology and is decreased in chronically inflamed gut blood vessels in inflammatory bowel disease (IBD; Crohn’s disease and ulcerative colitis). Mechanisms underlying decreased NO production in IBD gut microvessels are not fully characterized. Loss of NO generation may result from increased arginase (AR) activity, which enzymatically competes with nitric oxide synthase for the common substrate L-arginine. We characterized AR expression in IBD microvessels and endothelial cells and its contribution to decreased NO production. AR expression was assessed in resected gut tissues and human intestinal microvascular endothelial cells (HIMEC). AR expression significantly increased in both ulcerative colitis and Crohn’s disease microvessels and submucosal tissues compared with normal. TNF-α/lipopolysaccharide increased AR activity, mRNA and protein expression in HIMEC in a time-dependent fashion. RhoA/ROCK pathway, a negative regulator of NO generation in endothelial cells, was examined. The RhoA inhibitor C3 exoenzyme and the ROCK inhibitor Y-27632 both attenuated TNF-α/lipopolysaccharide-induced MAPK activation and blocked AR expression in HIMEC. A significantly higher AR activity and increased RhoA activity were observed in IBD submucosal tissues surrounding microvessels compared with normal control gut tissue. Functionally, inhibition of AR activity decreased leukocyte binding to HIMEC in an adhesion assay. Loss of NO production in IBD microvessels is linked to enhanced levels of AR in intestinal endothelial cells exposed to chronic inflammation in vivo.

endothelium; microvasculature; nitric oxide synthase

MICROVASCULAR DYSFUNCTION with a loss of endothelial generation of nitric oxide (NO) is present in chronically inflamed areas of human inflammatory bowel disease [IBD; Crohn’s disease (CD), ulcerative colitis (UC)]. Microvascular dysfunction in IBD is linked to impaired vasorelaxation and impaired tissue perfusion as well as sustained endothelial activation and altered patterns of leukocyte recruitment, both of which may worsen and perpetuate chronic inflammation (23, 30). Because blood vessels comprising the microvascular circulation play a central role in essentially all aspects of tissue physiology, including wound repair, immune regulation, and the inflammatory response, characterizing mechanisms underlying microvascular dysfunction represents an essential area for understanding chronic inflammatory disease pathogenesis, including IBD.

Microvascular dysfunction in IBD involves an alteration in nitrogen and oxyradical balance, where the microvascular endothelium fails to generate NO and instead produces sustained levels of superoxide anion (13). However, the mechanisms that underlie loss of endothelial NO generation in IBD gut microvessels are not completely defined. Previous work from our group has demonstrated a loss of nitric oxide synthase (NOS) 2 (NOS2) transcription in chronically inflamed IBD endothelial cells (25), but additional mechanisms can also be considered that do not directly involve NOS isoforms. When we consider that NO generation requires conversion of L-arginine (L-Arg) to citrulline by NOS enzymes, the availability of this substrate may play a key role in vascular homeostasis.

The arginase (AR) enzymes (ARI and ARII) convert L-Arg into urea and L-ornithine, precursors for polyamines and L-proline compounds, which are vital to tissue homeostasis and wound repair (11, 15). AR competes with inducible NOS (iNOS; NOS2), the high-output, inducible pathway for increased production of NO, for L-Arg, their common substrate in multiple cell types including endothelial cells. In cases in which L-Arg is limited, NO levels can fall, and this may be the result of enhanced AR activity (8). AR expression and activity can be regulated in many cell types, including vascular endothelial cells, smooth muscle cells, and macrophages, by various cytokines (32), and the two known isozymes of AR are either constitutively expressed or induced. In endothelial cells both isoforms ARI and ARII have been previously demonstrated (7). ARI or the hepatic isofrom is induced by hypoxia, lipopolysaccharide (LPS), and IL-13 in a variety of cells and tissues (19, 29). ARII, the extrahepatic isofrom, is also inducible by hypoxia, LPS, TNF-α, IFN-γ, and 8-bromo-cGMP (7, 27).

In addition to limiting NO generation, a central function of AR is the production of L-ornithine, a precursor for synthesis of polyamine and L-proline compounds that are vital to tissue repair following injury (11, 15). In inflammatory diseases, such as acute respiratory distress syndrome, NO production from L-Arg via NOS is involved in the initial host response, whereas L-ornithine production from L-Arg via AR is involved in healing (18). However, AR activity has never been measured in the human gastrointestinal microvasculature in relation to IBD.

We hypothesized that increased AR expression in IBD microvessels and endothelial cells contributes to decreased

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-NO production in chronically inflamed gut microvasculature. The aim of this study was to characterize the expression and enzymatic activity of AR in control and IBD endothelial cells, microvessels, and submucosal gut tissues to define molecular mechanisms underlying the loss of -NO generation.

MATERIALS AND METHODS

Patients. Normal colonic and small intestinal specimens were obtained from surgical margins from patients undergoing scheduled surgical bowel resections. These samples were used to derive control endothelial cultures and also constituted the control tissues used in this study. Small intestinal and colonic specimens from patients with CD and colonic specimens from patients with UC who underwent surgical resection comprised the IBD group. The degree of involvement was recorded, distinguishing uninvolved from involved areas of IBD. The use of human tissues and isolation of endothelial cells (HIMEC) from these tissues was approved by the Institutional Review Board of The Medical College of Wisconsin.

Reagents. Endothelial cell growth supplement was from Upstate Biotech (Lake Placid, NY). RPMI 1640 medium and FBS were obtained from BioWhittaker (Walkersville, MD). Human plasma fibronectin was purchased from Chemicon International (Temecula, CA). MCDB-131 medium, porcine heparin, and PSF (penicillin/streptomycin/fungizone) were from Sigma Chemical (St. Louis, MO). Rhoa, ROCKI, ROCKII, and ARII antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to ARI was from BD Biosciences, Transduction Laboratories (San Diego, CA). Enhanced chemiluminescence and all other electrophoresis reagents were from Bio-Rad (Hercules, CA). Unless otherwise indicated, all other chemicals used in this study were purchased from Sigma. AR inhibitor (S)-(2-boronoethyl)-L-cysteine-HCl (BEC) was obtained from Calbiochem.

HIMEC isolation. HIMEC were isolated and cultured as previously described (5).

Activation and pharmacological modulation of HIMEC. HIMEC activation was achieved following TNF-α (100 U/ml), LPS (1 µg/ml from Escherichia coli 0111:B4, Sigma), or IL-1β (100 U/ml) stimulation for specified time periods. All cytokines and chemokines used in this study were purchased from R and D Systems (Minneapolis, MN). By using pharmacological inhibitors, 10 µM of SB203580 (p38 MAPK), 10 µM of PD098059 (p44/42 MAPK), 5 µM of Clostridium botulinum C3 exoenzyme (Rhoa inhibitor, Calbiochem), and 10 µM of Y-27,632 Rho-associated coiled-coil kinase (ROCK inhibitor, Calbiochem), the signaling pathways involved in AR were defined.

Immunofluorescence staining. HIMEC monolayers were grown on coverslips to 80% confluency. Following HIMEC activation, monolayers were rinsed once in PBS, fixed with cold methanol for 30 min, and blocked with 5% BSA in PBS with Ca2+ and Mg2+ for 60 min. The arinsag was immunolocalized by using antibodies to ARI and ARII followed by incubation with a FITC-conjugated secondary antibody (Santa Cruz Biotechnology). Slides were mounted and visualized by using a fluorescence microscope (Olympus BX-40) and a Leica DFC 300FX camera.

Histological analysis. Full-thickness tissues were fixed in 4% (wt/vol) paraformaldehyde in PBS, saturated in 20% (wt/vol) sucrose in PBS, and embedded in optimum cutting temperature compound (OCT compound, Sakura, Osaka, Japan) as previously described (14). Frozen sections (7 µm) were prepared and stained with a tissue- and cell-staining kit (R and D Systems) and anti-ARI monoclonal antibody or mouse IgG as a negative control, respectively. After immunodetection using dianamobenzidine, sections were counterstained in Mayer’s hematoxylin and mounted. Positive staining appeared as a dark brown precipitate.

RNA extraction and semiquantitative RT-PCR. Arginase gene expression was assessed in submucosal tissues, unstimulated and activated confluent HIMEC cultures, with a combination of 100 U/ml TNF-α and 1 µg/ml LPS for 0, 6, 12, 24, and 48 h at 37°C. Total RNA was extracted by using TRizol LS Reagent (Invitrogen, Carlsbad, CA). Total RNA (2 µg) was reverse transcribed using SuperScript III (Invitrogen) in a total reaction volume of 20 µl, and 2 µl of reverse-transcription product (cDNA) were PCR amplified by using Taq DNA polymerase (Invitrogen) and 0.5 µl each of 10 µM arginase forward and reverse primers (Integrated DNA Technologies, Coralville, IA) (Table 1). β-Actin primers were included in the reaction as an internal control for the efficiency of the RT and the amount of RNA used in the RT-PCR. After an initial period of 5 min at 95°C, the PCR reactions were cycled 35 times (35 cycles for ARI and ARII, and 25 cycles for β-actin) between 95°C (denaturation) for 1 min; 55°C (annealing) for 1 min; and 72°C (extension) for 1 min. Samples were incubated at 72°C for an additional 5 min after the last cycle was completed. The primer sequences and PCR product sizes are shown in Table 1. PCR products were run on 1% agarose gels and stained with 0.5 µg/ml of ethidium bromide, visualized under UV light and photographed.

Western blot analysis. Gel electrophoresis and Western blot analysis were performed as previously described (26). Briefly, to extract total protein, tissue was first pulverized under liquid nitrogen, then homogenized (Brinkmann Instruments, Westbury, NY) in ice-cold buffer (HEPES 5 mM, pH 7.9, glycerol 26%, MgCl2 1.5 mM, EDTA 0.2 mM, DTT 0.5 mM, phenylmethylsulfonyl fluoride 0.5 mM) with NaCl (300 mM final) and incubated on ice for 30 min. After centrifugation twice at 15,000 g and 4°C for 20 min, protein concentration was determined by using a Bio-Rad protein assay, and the samples were stored at −80°C until use. Proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies against ARI (BD Transduction Laboratories), ARII and Rhoa (Santa Cruz Biotechnology) (1:500 dilution). Bound antibody was detected using secondary antibody tagged with horseradish peroxidase (1:20,000) (Santa Cruz Biotechnology) and visualized using Immun-Star (Bio-Rad) detection system.

Nitrite/nitrate assay. Confluent HIMEC cultures were activated with TNF-α/LPS for indicated time as above. Then cell culture media were removed for urea and nitrite/nitrate assays. HIMEC culture media were assayed in duplicate by using a chemiluminescence NO analyzer (Sievers model 280i, GE Analytical Instruments, Boulder, CO) according to manufacturer’s specifications. Briefly, 10 µl of sample was injected into a reaction chamber containing a vanadium (III) chloride and HCI mixture heated to 95°C to reduce nitrite, nitrate, and nitrosothiols to NO gas. The NO gas is pulled into the analyzer by a vacuum pump. The results were analyzed with a NaNO3 standard curve.

Urea assay. Urea production was assayed as a functional assessment of AR activity. For measurement of urea production from cultured cells, conditioned culture media was assayed in duplicate by using a QuantiChrom Urea Assay kit as above. Optical density was read at 520 nm on a 96-well plate analyzer (Thermo LabSystems). The results were expressed as nanograms of urea produced per minute. The detection range was 0.1 to 5 µg/ml. The QuantiChrom Urea Assay Kit was used as a control. Optical density was read at 520 nm on a 96-well plate analyzer (Thermo LabSystems).

Table 1. ARI, ARII, and β-Actin primers

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<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing (°C)</th>
<th>Extension (°C)</th>
<th>Product Size (bp)</th>
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<tr>
<td>Arginase I</td>
<td>Forward</td>
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<tr>
<td>Arginase II</td>
<td>Forward</td>
<td>422 bp</td>
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<td>Arginase I</td>
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manufacturer’s urea standard (50 mg/dl) was used to calibrate the analyzer.

Inhibition with L-Val. L-Valine (L-Val) was added at 3, 10, 30, and 100 mmol concentrations, along with 1 mmol L-Arg, given that L-Val is a competitive inhibitor of AR to cell cultures. The L-Arg concentration was chosen to approximate L-Arg levels in plasma (10, 19). Cells were then activated with TNF-α/LPS for 24 h and collected for Western blotting and RNA extraction. Medium was saved for urea and nitrite/nitrate assays.

Measurement of AR activity in tissues. Human intestinal tissue was obtained as described above, frozen in liquid nitrogen, and pulverized as previously described (26). The resulting tissue powder was homogenized in ice-cold buffer (1:4 wt/vol) containing 20 mM HEPES (pH 7.4) and 0.25 M sucrose in the presence of protease inhibitors (PMSF, leupeptin, aprotinin). The homogenate was centrifuged at 3,000  

Western blotting and RNA extraction. Medium was saved for urea and nitrite/nitrate assays.

Enzyme-linked immunosorbent assay (ELISA). TNF-α/LPS for 24 h and collected for

Fig. 1. Increased arginine (AR) activity in inflammatory bowel disease (IBD) submucosal tissues. Initial experiments examined AR activity in control and IBD tissues using full-thickness intestinal specimens. There was no difference in AR activity seen between control and IBD full-thickness specimens (both UC and CD specimens n = 5 for each; data not shown). In the next series of experiments, the submucosa was dissected from the full-thickness bowel and analyzed separately. The submucosa contains the rich vascular plexus of arterioles and venules that play a central role in the regulation of tissue perfusion in the bowel. When analyzed separately, significant differences between AR activity were seen in the submucosa of control and IBD patients (Fig. 1, A and B). AR activity in both UC (n = 5) and CD (n = 5) submucosal tissues was significantly higher than control submucosal samples (n = 5) from normal intestine. Analysis of -NO production from the submucosal specimens demonstrated a significant loss of -NO production in the IBD samples compared with controls, which follows the expected, reciprocal pattern compared with increased AR functional activity.

Enhanced AR mRNA expression in IBD submucosa. Next we determined the ARI and ARII mRNA expression in control and IBD submucosal specimens using semiquantitative RT-PCR.
and their respective primers. Levels of ARI, ARII, and β-actin mRNA were examined in control and IBD submucosal tissue homogenates, and densitometric analysis was performed after normalization to β-Actin, which served as an internal control. ARI mRNA (330 bp) expression was significantly increased in both CD and UC compared with controls (Fig. 2). Similar patterns of increased ARII mRNA (422 bp) expression were seen, with significantly increased expression of gene product in the IBD samples compared with controls (Fig. 2).

Enhanced AR protein expression in IBD submucosa. Next, levels of AR protein expression in control and IBD submucosal specimens were analyzed by Western blot analysis. As demonstrated in Fig. 3, A and B, level of ARI and ARII protein expression was significantly higher in submucosa homogenates from both CD and UC compared with controls.

AR immunostaining in control and IBD intestinal submucosal microvessels. The submucosa is comprised of several cell populations including vascular endothelium, smooth muscle, fibroblasts as well as infiltrating immune cells. To address the cellular sources of AR expression in the submucosa, tissue immunolocalization was performed. Initial experiments examined ARI expression in human gastrointestinal submucosa by immunohistochemistry. As shown in Fig. 4A, c and d, ARI was readily detected in IBD (both CD and UC) submucosal intestinal microvessels but not in normal control tissue. No specific immunostaining was observed by using the appropriate IgG isotype control antibody Fig. 4Ab. Then we complemented these results with a second series of experiments that examined the presence of ARI in submucosal tissues by immunofluorescence staining. Figure 4B, c and d demonstrates strong AR staining in IBD tissues but not normal tissues. Of note, the ARII antibody available at this time was not suitable for immunohistochemistry.

Inflammatory activation of HIMEC enhances expression of AR mRNA and protein. To determine whether AR expression in the gut specific microvascular endothelium was inducible, initial experiments were performed on HIMEC monolayers treated with classic inflammatory mediators known to be involved in IBD chronic inflammation for 24 h. Experiments evaluating mRNA expression by semiquantitative RT-PCR demonstrated increased ARI and ARII gene product following activation with IL-1β, and combinations of TNF-α/LPS and IL-1β/TNF-α (Fig. 5A). Activation of HIMEC with LPS alone resulted in very little to no increase in gene product for both AR isoforms.

We confirmed these findings with Western blot analysis. Again, HIMEC monolayers stimulated with IL-1β alone, as well as IL-1β/TNF-α, demonstrated increased protein expression of both ARI and ARII above baseline (Fig. 5B).

TNF-α/LPS stimulates AR activity in HIMEC. To confirm that the microvascular endothelium was a source of AR activity in the gut, experiments examining AR activity were performed using cultures of HIMEC isolated from control and IBD patients. AR activity in the HIMECs was measured by colorimetric determination of urea formed from L-Arg as described previously. HIMECs were assayed unstimulated and following

Fig. 2. Enhanced AR mRNA expression in IBD submucosa. Semiquantitative RT-PCR demonstrate that mRNA for both AR enzymes ARI and ARII were increased 2- to 3-fold in both CD and UC submucosal tissues compared with controls. β-Actin served as an internal control. Representative result from a total of 5 experiments performed on independent patient samples.

Fig. 3. Enhanced AR protein expression in IBD submucosa. Western blot analysis of submucosal specimens revealed upregulation of AR proteins in IBD tissues. A: increased level of ARI protein was higher in submucosal homogenates from both UC and CD compared with controls. B: similarly increased level of ARII protein was higher in submucosal homogenates from both UC and CD compared with controls. Images are representative gels from 5 independent experiments for each densitometry graph.
Fig. 4. AR immunostaining in intestinal submucosal microvessels. A: immunohistochemical localization of ARI in normal and IBD frozen sections using monoclonal antibody against ARI. ARI was readily detected in IBD (both CD and UC) submucosal intestinal microvessels but not in normal control tissues (c and d). No specific immunostaining was observed by using the appropriate IgG isotype control antibody (b). CD31 immunostaining was used as a positive control (a). Representative sections of 5 independent experiments are shown. Original magnification ×400. B: immunofluorescence staining of ARI in submucosal tissues. Strong ARI staining in IBD tissues but not normal tissues (c and d) was detected. Of note the ARII antibody was not suitable for immunohistochemistry. Images are representative examples from 5 independent experiments.
activation with 100 U/ml of TNF-α and 1 μg/ml of LPS for different time points from 1 to 48 h. AR activity increased to more than twofold increase above control (n = 5). A significant increase in AR activity was detected at 18 h, which maximized at 24 h following TNF-α/LPS activation in HIMECs (Fig. 6).

**Fig. 6.** TNF-α/LPS stimulates AR activity in HIMEC. To confirm that the microvascular endothelium was a source of AR activity in the gut, AR activity was measured by using cultures of HIMEC isolated from control and IBD patients. AR activity in HIMEC following 100 U/ml of TNF-α and 1 μg/ml of LPS was increased by >2-fold above the control (n = 5). A significant increase in AR activity was detected at 18 h maximized at 24 h following TNF-α/LPS activation. Graph is means ± SD from 3 independent experiments.

**Fig. 7.** TNF-α/LPS enhances AR mRNA and protein expression in HIMEC. A: semiquantitative RT-PCR demonstrate that ARI mRNA expression was increased 2- to 3-fold in TNF-α/LPS-activated HIMEC by 12–24 h, compared with unstimulated control HIMEC. Similarly, the ARII mRNA expression was significantly increased following TNF-α/LPS activation of HIMEC by 24 h. β-Actin mRNA expression showed comparable density of bands for both control and activated HIMEC. Image is representative of 4 independent experiments. B: Western blot analysis of ARI and ARII protein expression in HIMEC following TNF-α/LPS. Both ARI and ARII protein expression were enhanced in activated cells compared with basal, unstimulated HIMECs. The AR protein expression in HIMEC was time dependent and maximized for both ARI and ARII by 24 h; however, the ARII protein level decreased by 48 h whereas ARI did not. Image is representative of 3 independent experiments.

**Fig. 5.** Enhanced AR mRNA and protein expression in human intestinal microvascular endothelial cells (HIMEC). Inflammatory activation of HIMEC and increased expression of AR by various activators. A: semiquantitative RT-PCR demonstrate the increased ARI and ARII by IL-1β, TNF-α, and combination of these cytokines in activated HIMEC. LPS, lipopolysaccharide. B: Western blot analysis of HIMEC lysates demonstrate an increased expression of ARI and ARII protein by IL-1β and combined IL-1β/TNF-α. Image is representative result of 5 independent experiments for both RT-PCR and Western blotting.

**Fig. 6.** TNF-α/LPS stimulates AR activity in HIMEC. To confirm that the microvascular endothelium was a source of AR activity in the gut, AR activity was measured by using cultures of HIMEC isolated from control and IBD patients. AR activity in HIMEC following 100 U/ml of TNF-α and 1 μg/ml of LPS was increased by >2-fold above the control (n = 5). A significant increase in AR activity was detected at 18 h maximized at 24 h following TNF-α/LPS activation. Graph is means ± SD from 3 independent experiments.

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TNF-α/LPS enhanced AR protein expression in HIMEC. AR protein expression in HIMEC following TNF-α/LPS activation was determined by Western blot analysis. As demonstrated in Fig. 7B, the amount of both AR1 and ARII protein was significantly higher following activation compared with basal, unactivated HIMECs. The AR protein expression in HIMEC was time dependent and maximized for both AR1 and ARII by 24 h; however, the ARII protein level decreased by 48 h whereas AR1 did not.

Arginase immunolocalization in HIMEC. Next, we determined the AR immunolocalization in HIMEC, as shown in Fig. 8A. Immunofluorescent staining of resting HIMEC demonstrated very little to no AR immunoreactivity (Fig. 8A); however, following TNF-α/LPS activation, cytosolic expression of AR1 and ARII was evident compared with control cells (Fig. 8A, c and d). The TNF-α/LPS activated cells demonstrate the fluorescent staining in the cytoplasmic region of mature, spindle-shaped cells.

AR immunolocalization was then evaluated in HIMEC following IL-1β stimulation. Similar to the pattern demonstrated for TNF-α/LPS, IL-1β induced a potent activation of both AR1 and ARII protein staining in the cytoplasm of the activated HIMEC (Fig. 8B, c and d).

Involvement of MAPK signaling pathways in AR expression in HIMEC. The next series of experiments examined the relationship between HIMEC expression of AR and the MAPK signaling cascades. We have previously defined that the MAPK signaling cascades are mediated by TNF-α/LPS activation in HIMEC (25). Using Western blotting of immunoprecipitated cell lysates with specific phospho-antibodies [p44/42 MAPK (ERK1/2), p38 MAPK, and JNK/SAPK] we confirmed the activation of the three MAPK family members (Fig. 9A). Time-course experiments demonstrated that p38 MAPK underwent a rapid and transient activation, which peaked at 15 min, whereas p42/44 MAPK activation following TNF-α/LPS stimulation peaked at 30 min.

Next, we examined the involvement of the MAPK signaling pathways in AR expression following TNF-α/LPS activation of HIMEC. The p38 MAPK and p44/42 MAPK inhibitors (SB203580 and PD98059) failed to inhibit the TNF-α/LPS induced expression of AR1, which was decreased with ARII siRNA gene silencing (Fig. 9B). In contrast, TNF-α/LPS induced expression of ARI was attenuated in response to both SB203580 and PD98059, as well as ARII siRNA (Fig. 9B).

To determine the role of RhoA as a mediator of MAPK activation in TNF-α/LPS activated HIMEC, we used the selective Rho inhibitor C3 exoenzyme. We found that inhibition of the Rho pathway by C3 exoenzyme significantly reduced both p38 MAPK and p44/42 MAPK activity in HIMEC (Fig. 9C). Next, we examined the role of ROCK, a downstream effector of Rho in TNF-α/LPS-mediated activation of HIMEC. Similar to Rho inhibitor C3 exoenzyme, the ROCK inhibitor Y-27632 exerted an inhibitory effect on both p38 MAPK and p44/42MAPK (Fig. 9C). The inhibition of p38 MAPK by RhoA and ROCK siRNA implied that several homeostatic mechanisms in HIMEC in addition to AR would also be linked to these pathways.

Involvement of MAPKs signaling pathways in AR activity in HIMEC. To investigate the possible involvement of the MAPK signaling pathways in AR activity, HIMECs were pretreated with respective inhibitors for 30 min before TNF-α/LPS activation. TNF-α/LPS-induced AR activity (i.e., urea production) was not attenuated by either SB203580 (selective p38 MAPK inhibitor) or PD98059 (specific p42/p44 MAPK inhibitor) (Fig. 10A). Because HIMEC activation with TNF-α/LPS is known to induce NOS2, the high-output pathway of NO generation, we evaluated the production of NO metabolities generated from HIMEC monolayers using chemiluminescence. SB203580 significantly decreased NO generation from TNF-α/LPS activated HIMEC, which was not seen with PD09059 (Fig. 10B). These data confirm previous reports from our laboratory that link NOS2 gene expression to p38 MAPK activation in HIMEC stimulated with TNF-α/LPS (25). These new observations regarding AR expression in conjunction with our past work defining NOS2 expression suggest that targeting p38MAPK may selectively shift the balance of l-Arg metabolism away from NO generation in activated HIMEC.

Effect of l-Val and l-Arg on NO production by HIMEC. The addition of l-Val at various concentrations (3–100 mM) to TNF-α/LPS activated HIMEC was used to assess inhibition of AR activity. Conditioned media from HIMEC incubated in the presence of increasing concentrations of l-Val resulted in significantly less urea production (Fig. 11A). The decrease in urea production was associated with a reciprocal increase in NO production. To confirm that these findings were the result of selective enzymatic inhibition, experiments were performed using both l-Val in combination with excess substrate (l-Arg) in both unstimulated and TNF-α/LPS-activated HIMECs. In these experiments, the addition of excess l-Arg to the HIMEC incubation medium resulted in significantly greater urea and NO production (Fig. 11B). Again, the presence of l-Val decreased urea generation and resulted in a reciprocal increase in NO metabolites.

Role of RhoA/ROCK signaling in AR expression in activated HIMEC. To determine whether AR expression is linked to intracellular signaling through RhoA and ROCK in TNF-α/LPS activated HIMEC, experiments were performed using the RhoA inhibitor C3 exoenzyme (5 μM) and the ROCK inhibitor Y-27632 (10 μM) at concentrations previously shown to inhibit RhoA or ROCK (2). Both agents demonstrated an inhibitory effect on AR1 and ARII mRNA (Fig. 12A). Western blotting was performed to confirm that RhoA/ROCK signaling was linked to AR protein expression in the activated HIMEC. C3 exoenzyme exerted a more potent inhibitory effect on ARII protein expression compared with Y-27632. Both inhibitors decreased expression of ARII protein levels (Fig. 12B). These findings demonstrate that signaling through RhoA/ROCK pathways plays a key role in AR expression in TNF-α/LPS activated HIMECs.

Increased RhoA activity in IBD submucosal tissues and HIMEC. Previously published data have demonstrated increased RhoA activity in CD mucosal tissues, as well as 2,4,6-trinitrobenzene sulfonic-induced colitis in the rat, which was ameliorated with the ROCK inhibitor Y-27632 (28). To investigate whether increased Rho activity is present in IBD patient submucosal tissues, experiments were performed using a Rho kinase activity assay on surgically resected tissues. Significantly increased RhoA kinase activity was demonstrated in both CD and UC submucosal samples, which were significantly higher (two- to threefold) compared with non-IBD intestinal controls (Fig. 12C). To confirm that increased Rho activity follows TNF-α/LPS activation of HIMEC, experi-
Fig. 8. AR immunolocalization in HIMEC. A: immunofluorescence staining of resting HIMEC demonstrated negligible AR immunoreactivity (b); however, following TNF-α/LPS activation, cytosolic expression of ARI and ARII was evident compared with control cells (c and d). The TNF-α/LPS-activated cells demonstrate the fluorescent staining in the cytoplasmic region of mature, spindle-shaped cells. Image is representative of 5 independent experiments. B: immunofluorescence staining of HIMEC following IL-1β activation, demonstrate cytosolic expression of ARI and ARII (c and d) compared with control cells (b). Image is representative of 5 independent experiments.
ments were performed using the Rho kinase activity assay. TNF-α/LPS significantly increased activated Rho, which was inhibited by both C3 exoenzyme as well as Rho siRNA (Fig. 12).

**HIMEC-leukocyte adhesion assay.** Lastly, we examined the functional significance of AR inhibition in gut microvascular endothelial cells. Previous work from our laboratory has demonstrated that inhibition of *NO production in HIMEC resulted in significant increases in leukocyte binding using in vitro static and flow adhesion assays (3). Unstimulated HIMEC bound low levels of U-937 monocyte-like cells (Fig. 13Aa) that readily increased with overnight TNF-α/LPS activation by a static endothelial-leukocyte adhesion assay (Fig. 13Ab). Pretreatment of HIMEC with L-Val and BEC did not affect the adhesion of U-937 leukocytes to unstimulated HIMEC monolayers (Fig. 13A, c and e). In marked contrast, both L-Val (30 mM) and BEC (8 μM) pretreatment of the TNF-α/LPS activated HIMEC demonstrated a dramatic decrease in U-937 binding (Fig. 13A, d and f). Quantification of enhanced leukocyte binding by TNF-α/LPS demonstrated a significant increase which was decreased by both AR inhibitors L-Val and BEC (Fig. 13B).

**DISCUSSION**

Microvascular dysfunction is present in the intestinal arterioles in human IBD and is linked to a loss of *NO generation by the microvascular endothelium. In the present study we have demonstrated that depletion of the NOS substrate L-Arg through the action of increased AR expression may contribute to the loss of *NO generation in IBD microcirculation and microvascular dysfunction. We report that both ARI and ARII have increased gene expression and activity in chronically inflamed IBD gut submucosal tissues. Furthermore, we have demonstrated that ARI and ARII gene expression, protein expression, and activity increases in isolated intestinal microvascular endothelial cells stimulated with proinflammatory mediators associated with IBD (i.e., TNF-α and IL-1β). These data suggest that increased expression of AR isozymes will diminish microvascular endothelial access to L-Arg, the key substrate required for generation of *NO, and this may be a key mechanism involved in the microvascular dysfunction associated with IBD.

Previous studies have indicated an important role of increased AR activity in endothelial pathobiology in human and animal models of disease. Even a moderate increase of 1.5- to 2-fold of AR in endothelial cells was felt to contribute to endothelial dysfunction in aged rats and in human studies examining diabetic erectile dysfunction (6). Xu et al. (31) demonstrated increased ARII expression in pulmonary endothelial cells isolated from patients with primary pulmonary hypertension, a fatal disease of unknown etiology that is characterized by low levels of pulmonary *NO. Although the majority of studies suggest that *NO levels may be increased in both CD and UC patient intestine during disease activity, the
localization of NO generation is not uniform. Our group has previously reported microvascular dysfunction and a loss of NO generation in the microvascular endothelium from areas of chronically inflamed IBD intestine (4, 13, 25). The approach we employed in the present study, focusing on submucosal tissues in addition to full-thickness specimens, demonstrated a loss of NO generation in the area of the intestine that contains the richest vascular plexus. Therefore, in concert with our previous findings demonstrating diminished NOS2 expression and activity in IBD derived endothelial cells, increased AR expression in the submucosa appears to be an additional mechanism which will contribute to microvascular dysfunction in IBD.

Recently, AR has gained attention in studies focusing on the pathophysiology of IBD. Gobert et al. (11) demonstrated that

Fig. 10. Effect of MAPK inhibitors on AR activity in HIMEC. A: urea production was not attenuated by either SB203580 (selective p38 MAPK inhibitor) or PD98059 (specific p42/p44 MAPK inhibitor) in TNF-α/LPS activated HIMEC. B: SB203580 but not PD98059 significantly decreased NO generation in TNF-α/LPS-activated HIMEC. L-Val, L-valine; L-Arg, L-arginine. Data are expressed as means ± SD from a total of 5 independent experiments.

Fig. 11. Effect of L-Val and L-Arg on NO production by HIMEC. A: addition of L-Val at various concentration (3–100 mM) to TNF-α/LPS-activated HIMEC was used to assess inhibition of AR activity. The decrease in urea production was associated with a reciprocal increase in NO production. Data are means ± SD from a total of 5 independent experiments. B: L-Val in combination with excess substrate (L-Arg) in both unstimulated and TNF-α/LPS-activated HIMECs resulted in significantly greater urea and NO production. Again, the presence of L-Val decreased urea generation and resulted in a reciprocal increase in NO metabolites. Data are expressed as means ± SD from a total of 3 independent experiments.
AR exerts a protective role in the Citrobacter rodentium model of colitis. In this murine system, ARI was increased in mice with colitis in the gut epithelium, and supplementation of animals with l-Arg in combination with NOS2 inhibition resulted in significant improvement in both colitis and animal survival. The authors hypothesized that, in this mouse model of colitis, inhibition of NOS2 and shunting of l-Arg through AR would result in an increased production of ornithine, which is acted on by ornithine decarboxylase enzymes to produce polyamines. Polyamines, in turn, are felt to play a key role in tissue repair and wound healing, which mediated in part the improvement of the colitis. Although the results from this study of rodent colitis appear opposite of our findings, we may consider several explanations and propose a dual role for AR in chronic gut inflammation. Our study focused on the role of AR in intestinal microvascular endothelial dysfunction and the loss of NO generation, which was not the focus of Gobert’s work (11), who primarily examined epithelial AR expression and did not specifically examine microvascular physiology. Given that NO plays a key anti-inflammatory role in vascular physiology (3, 16), a dual role for AR as both anti-inflammatory (in the microvasculature) as well as proinflammatory (in epithelium and macrophages) depends largely on the specific cellular expression in tissues. Secondly, murine tissues produce significantly larger quantities of NO compared with human samples, where NO appears to play a larger role as an endogenous antioxidant and signaling molecule. Therefore, studies in human and animal systems must consider the significant differences in NO production that are present during normal homeostasis as well as during disease physiology.

A novel investigative strategy employed in this study was the analysis of submucosal specimens from control and IBD bowel. Whole thickness intestinal specimens failed to show alterations in AR function from IBD tissues, whereas isolated submucosal specimens demonstrated significant increases in AR. These data highlight the importance of cellular localization of AR and its potential contribution to microvascular dysfunction in IBD. The analysis of submucosal specimens removed the potential contribution of epithelial cells in our characterization of AR in control and IBD bowel.

The AR enzymes are present in various cell types, including endothelial cells, macrophages, and hepatocytes (7, 12, 22, 24, 32). In porcine coronary arterioles and rat aortic endothelial cells, ARI is constitutively expressed (7, 32), whereas ARII is inducible by LPS and cytokines (7). In bovine pulmonary microvascular endothelial cells, both ARI and ARII were inducible with TNF-α and LPS stimulation (9). In human lung microvascular endothelial cells, both ARI and ARII isoforms have been demonstrated, with ARII found to be increased in patients with primary pulmonary hypertension (31). Our data also demonstrate the expression of both ARI and ARII mRNA in submucosal arteriolar endothelium in control intestine, with marked increase in IBD intestinal endothelium. In human gut-specific microvascular endothelial cells, both ARI and ARII were found to be inducible in response to TNF-α and LPS.

Following TNF-α/LPS activation, ARI and ARIII gene and protein expression as well as activity was significantly increased compared with inactivated HIMEC. When AR expression from HIMEC derived from normal bowel was compared with IBD derived HIMEC was examined, no appreciable

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**Fig. 12. Role of RhoA/ROCK in AR expression in HIMEC and IBD submucosal tissues.**

A: AR expression is linked to RhoA and ROCK in TNF-α/LPS activated HIMEC. C3 exoenzyme Rho inhibitor and Y-27632 ROCK inhibitor demonstrated an inhibitory effect on ARI and ARII mRNA. B: Western blotting demonstrates that C3 exoenzyme exerted a more potent inhibitory effect on ARI protein expression compared with Y-27632. Both inhibitors decreased expression of ARII protein in TNF-α/LPS activated HIMECs. C: RhoA kinase activity assay on surgically resected submucosal tissues demonstrate that RhoA activity was significantly increased (2- to 3-fold) in both CD and UC patients compared with non-IBD intestinal controls. D: TNF-α/LPS significantly increased RhoA activity in HIMEC, which was inhibited by both C3 exoenzyme as well as Rho siRNA. Images are representative from 3 independent experiments.
differences in either constitutive or inducible expression were detected. This finding was in contrast with our previously published finding that NOS2 expression is silenced in IBD-derived HIMEC (4). Although we did not find long-lived disease-specific alterations in AR expression in HIMEC derived from IBD patients, the data in this study suggest that increased AR expression and function in IBD microvessels is directly related to the presence of local inflammatory mediators (i.e., TNF-α, IL-1β) that are known to be elevated in the chronically inflamed bowel. Our previous studies have also shown that NOS3 gene and protein expression did not modulate with cytokine or LPS in HIMEC, and no disease-specific differences between control and IBD endothelial cultures were identifiable (4).

One important strength of our work was the use of organ-specific microvascular endothelial cells to define the expression and function of AR. Our findings suggest that increased urea production in HIMEC is TNF-α/LPS activated, at least in part, due to increased expression of ARI and ARII. Moreover, inhibition of AR resulted in an increased NO production via an increase in bioavailability of L-Arg to NOS. Together these data support our hypothesis that loss of NO production in IBD microvessels (both chronically inflamed CD and UC) is linked to enhanced levels of AR in endothelial cells exposed to chronic inflammation in vivo and suggest that NOS and AR compete for a common substrate pool of L-Arg.

Our studies demonstrated that potent inflammatory activation of HIMEC with TNF-α/LPS resulted in an increase in
production of both \(\cdot\text{NO}\) metabolites and an increase in urea production starting at 4 and 6 h, respectively. Thus it appears that TNF-\(\alpha\)/LPS rapidly upregulate both NOS and AR in HIMEC. However, the time course of production via these two competing enzymatic pathways varied, with \(\cdot\text{NO}\) production rising first and urea production peaking later at 24 h. These studies suggest that the biological effect of \(\cdot\text{NO}\) is more immediate in this endothelial cell system, whereas the effect of AR would function to reverse the action of increased \(\cdot\text{NO}\) bioavailability.

Compelling data suggest that in the vasculature the small G protein RhoA plays an important role in regulation of various cellular functions (2, 17). It has been shown that RhoA, via its downstream effector ROCK, plays an important role in regulation of endothelial NOS (eNOS) gene expression and enzyme activity that may affect endothelial function at multiple levels (21). The role of RhoA/ROCK pathway in inhibition of Akt/eNOS cascade, i.e., activation of RhoA/ROCK inhibits PKB/Akt activity and subsequently activation or phosphorylation of eNOS at S-1177, has been shown (21). The involvement of RhoA/ROCK pathway in the regulation of AR activity and expression in human umbilical vein endothelial cells and in endothelial dysfunction in atherosclerosis has been shown (20).

The importance of Rho activation in human CD and animal models of IBD was established by Segain and colleagues (28). These authors demonstrated increased Rho kinase activity in patient material as well as a rat model of colitis, which improved clinically following treatment with Y-27632, an inhibitor of ROCK, a downstream molecule in the Rho signaling cascade. These authors evaluated mucosal samples and demonstrated that Rho plays a key role in the activation of IkB and expression of TNF-\(\alpha\). Our data suggest that Rho activation may also be involved in submucosal tissues in IBD, in which additional mechanisms, including increased AR expression in the microvasculature, can contribute to chronic inflammation.

We evaluated several strategies to inhibit the effect of AR. L-Val is a competitive inhibitor of AR, and our studies in HIMEC demonstrated a dose-dependent effect of this compound on decreased AR activity. Use of L-Val resulted in preferential shunting of L-Arg consumption through NOS and gave an indirect measure of NOS activity in our HIMEC system. Additional studies were performed using inhibitors of signaling pathways involved in the expression of AR. Neither the p38 MAPK inhibitor SB203580 nor the p44/42 MAPK inhibitor PD098059 altered AR function and activity in TNF-\(\alpha\)/LPS-stimulated HIMEC. The C3 exoenzyme, a derivative of Clostridium botulinum toxin, inhibited AR expression and activity in HIMEC. C3 exoenzyme functions by inactivating RhoA by ADP ribosylation (1). Moreover, inhibition of ROCK, a downstream effector of RhoA, by Y-27632 abolished the effect of TNF-\(\alpha\)/LPS, suggesting that AR activity in HIMEC is through the RhoA/ROCK pathway. These studies using pharmacological inhibitors were confirmed by siRNA gene silencing for RhoA signaling molecules in HIMEC. The importance of RhoA and ROCK signaling in AR expression was further supported by the experiments showing increased RhoA kinase activity in submucosal homogenates from UC and CD patients compared with normal control. Furthermore, the higher AR activity in the IBD samples and TNF-\(\alpha\)/LPS activated HIMEC was associated with higher RhoA activity, suggesting a role for RhoA in AR activity.

The functional significance of \(\cdot\text{NO}\) production in the microvascular endothelium has been substantiated by a number of studies and has been confirmed in human gut microvascular endothelial cells (3, 16). Our studies using two pharmacological strategies to inhibit AR in HIMEC demonstrated that inhibition of this enzymatic pathway significantly decreased leukocyte binding at a 24-h time period. The presumed mechanism involves an increase in \(\cdot\text{NO}\) availability, which functions to quench intracellular superoxide anion, a key mechanism of HIMEC activation (3). In preliminary experiments, we saw no direct effect of the AR inhibitor L-Val on HIMEC expression of NOS2 by use of Western blotting (data not shown). The significance of these experiments suggest that inhibition of vascular endothelial AR may function as a strategy to decrease leukocyte-endothelial interaction and potentially ameliorate inflammation in IBD.

Our data demonstrate the complex interplay between AR and NOS in HIMEC. The importance of p38MAPK in the expression of AR as well as NOS2 suggests that intertwining signaling pathways will be involved in the regulation of arginine metabolism in HIMEC. In conclusion, RhoA/ROCK pathway plays a role in AR activity in HIMECs, and the increased AR activity was associated with higher RhoA expression in both IBD tissue and TNF-\(\alpha\)/LPS-activated HIMEC. Thus targeting arginase in the vasculature may represent a novel therapeutic strategy for treatment of IBD.

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