Proteasome Inhibition Attenuates Nitric Oxide Synthase Expression, VCAM-1 Transcription and the Development of Chronic Colitis

ELAINE M. CONNER, STEPHEN BRAND, JONATHAN M. DAVIS, F. STEPHEN LAROUX, VITO J. PALOMBELLA, JOHN W. FUSELER, DAVID Y. KANG, ROBERT E. WOLF and MATTHEW B. GRISHAM

Departments of Molecular and Cellular Physiology (E.M.C., J.M.D., F.S.L., D.Y.K., M.B.G.) and Medicine (J.W.F., R.E.W.), Louisiana State University Medical Center, Shreveport, Louisiana, and ProScript, Inc., Cambridge, Massachusetts (S.B., V.J.P).

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ABSTRACT

The objectives of this study were to (1) assess the role of the 26S proteasome complex in regulating the expression of the inducible isoform of nitric oxide synthase (iNOS) and vascular cell adhesion molecule-1 (VCAM-1) in a model of chronic granulomatous colitis in vivo and (2) determine the role of the proteasome in regulating the inflammatory response observed in this model of chronic gut inflammation. The selective proteasome inhibitor MG-341 (0.3 mg/kg) was administered by gavage beginning immediately before the induction of colitis and continuing daily thereafter for the entire 14-day experimental period. We found that chronic proteasome inhibition using MG-341 significantly attenuated the peptidoglycan/polysaccharide (PG/PS)-induced up-regulation of iNOS in the colon and spleen and the consequent increase in plasma levels of nitrate and nitrite. Furthermore, we found that the proteasome inhibitor suppressed the up-regulation of the adhesion molecule VCAM-1 in the colon. We also found that MG-341 attenuated PG/PS-induced increases in macroscopic colonic inflammation, bowel wall thickness, colonic dry weight and colonic MPO activity. Treatment with MG-341 also significantly reduced PG/PS-induced increases in macroscopic spleen inflammation, spleen weight and spleen MPO activity. We conclude that the 26S proteasome complex plays an important role in regulating the PG/PS-induced up-regulation of iNOS and VCAM-1 in vivo and appears to be important in regulating colonic and splenic inflammation.

It is becoming increasingly apparent that chronic intestinal and/or colonic inflammation is associated with the sustained overproduction of NO. For example, it has been demonstrated that the chronic gut inflammation induced by exogenous agents (Grisham et al., 1994; Hogaboam et al., 1995; Miller et al., 1992) or the spontaneous colitis observed in genetically engineered rodents (Aiko and Grisham, 1995; Berg et al., 1996), nonhuman primates (Ribbons et al., 1995) or human IBD (ulcerative colitis, Crohn’s disease) (Boughton-Smith et al., 1993; Middleton et al., 1993) is characterized by the enhanced expression of the inducible isoform of NO synthase (iNOS) and increased production of NO. Furthermore, pharmacological intervention studies have demonstrated that NO or NO-derived metabolites mediate at least some of the pathophysiology associated with a variety of models of chronic gut inflammation (Grisham et al., 1994; Hogaboam et al., 1995; Miller et al., 1992; Rachmilewitz et al., 1995).

Recent work by Xie et al. (1994) has unequivocally shown that the expression of iNOS is regulated by NF-κB. NF-κB is a ubiquitous transcription factor and pleiotropic regulator of numerous inflammatory and immune responses. Once activated, NF-κB translocates to the nucleus of the cell, where it binds to its consensus sequence on the promoter-enhancer region of different genes and up-regulates the expression of a variety of proinflammatory cytokines, adhesion molecules and enzymes (Collins et al., 1995; Laio et al., 1995; Xie et al., 1994). NF-κB belongs to the Rel family of transcription factors (Baueuerle and Henkle, 1994; Kopp and Ghosh, 1994) in which members share a region of ~300 amino acids known as the Rel homology domain. The heterodimeric NF-κB is composed of p50 and p65 subunits and is normally sequestered in the cytoplasm as an inactive complex associated with its inhibitor IκB (Baueuerle and Baltimore, 1988). It is thought that multiple signaling pathways converge to enhance reactive oxygen metabolism within the cell. This intracellular

ABBREVIATIONS: PG/PS, peptidoglycan/polysaccharide; MPO, myeloperoxidase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; IBD, inflammatory bowel disease; VCAM, vascular cell adhesion molecule; IκB, inhibitor-κB; ICAM, intercellular adhesion molecule; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; NF-κB, nuclear factor-κB.
oxidative stress has been proposed to activate one or more redox-sensitive kinases that specifically phosphorylate IκB (i.e., IκBα). Once phosphorylated, IκB is selectively ubiquitinated. The polyubiquitinated IκB subunit is then selectively degraded by the nonlysosomal, ATP-dependent 26S proteasome complex (Bog et al., 1993; Brown et al., 1995).

Until very recently, the physiological significance of the 26S proteasome has been limited by the lack of selective inhibitors of this proteolytic complex. Several recent studies using selective proteasome inhibitors have proven useful in identifying the ubiquitin-proteasome pathway as an important route for NF-κB activation in vitro (Adams and Stein, 1996; Palombella et al., 1994; Traenckner et al., 1994). Indeed, Griscavage et al. (1996) have demonstrated that the proteasome pathway is essential for the upregulation of iNOS in vitro. It should be noted that the proteasome-dependent activation of NF-κB is also known to transcriptionally activate the expression of a number of different proinflammatory cytokines and adhesion molecules such as ICAM-1, E-selectin and VCAM-1 (Adams and Stein, 1996). In-vivo colonic inflammation has made it possible to test whether this multisubunit complex is active and selective inhibitors of the 26S proteasome has proven useful in reducing all nitrate (NO$_3^-$) to nitrite (NO$_2^-$) using selective proteasome inhibitors have proven useful in identifying the ubiquitin-proteasome pathway as an important route for NF-κB activation in vitro (Adams and Stein, 1996).

**Materials and Methods**

Reagents. PG/PS derived from group A streptococci was obtained as a sterile, endotoxin-free solution (3.2 mg rhamnose/ml; Lee Labs., Grayson, GA) and the selective proteasome inhibitor MG-341 was supplied by ProScript Inc. (Cambridge, MA). MG-341 is a newly developed, orally active dipeptide boronate derivative that possesses an IC$_{50}$ value for the 26S proteasome of ~0.6 μM (Adams and Stein, 1996).

Induction of colitis. Specific pathogen-free female Lewis rats (175–199 g) were housed in shoebox cages and given free access to water and standard laboratory rat chow. A total of 30 rats were randomized into four groups consisting of an untreated saline-injected control group ($n = 5$), saline-injected control rats treated with 0.3 mg/kg/day MG-341 orally for 14 days ($n = 6$), PG/PS-treated rats given 0.2 ml of 0.5% methylecellulose/day orally for 14 days ($n = 10$) and PG/PS-treated rats given 0.3 mg/kg/day MG-341 orally for 14 days ($n = 9$). Preliminary studies demonstrated that MG-341 at 0.3 mg/kg/day was the minimum dose required to inhibit the expression of iNOS in vivo.

Animals were anesthetized via inhalation of isoflurane (Aerrane; Ohmeda PPD Inc, Liberty Cornet, NJ), and their descending colons were exposed by laparotomy using aseptic technique. Colitis was induced via 10 to 12 intramural (subserosal) injections (40–50 μl/injection) of PG/PS (12.5 μg rhamnose/g b.wt.) into the distal colon (4 cm) using a 30-gauge needle (Yamada et al., 1993). Control animals were treated identically using 10 injections (40–50 μl) of a sterile saline solution. Rats were allowed to recover from surgery and given free access to food and water. On the day of surgery, one half of the drug dose was administered by gavage (suspended in 0.5% methylcellulose) 2 hr before surgery with the remaining dose administered 4 hr after surgery and then once daily thereafter for the entire 14-day experimental period.

Macroscopic, histological and biochemical evidence of inflammation. Two weeks after the induction of colitis, animals were anesthetized with Inactin (Research Biochemicals, Natick, MA) after a 24-hr fast. The descending aortas were exposed, and a 3-ml aliquot of blood was obtained using 3.8% sodium citrate as anticoagulant. The descending colons were exposed, and macroscopic inflammation was assessed according to the criteria described in Table 1. In addition, the incidence of extraintestinal manifestations, such as the appearance of spleen nodules and adhesions, was noted and recorded. The length and weight of the involved segment of colon were recorded, and tissues were obtained for histology, wet-to-dry ratios and MPO activity determination. Spleen weights were also recorded. Wet weights of colonic samples were recorded, as well as their dried weights after a 48-hr incubation at 80°C. For histological analysis, samples were fixed in 2% paraformaldehyde, dehydrated and embedded in JB-4 (Polysciences, Warrington, PA). Two-micrometer sections were cut transversely and stained with hemotoxylin and eosin. Bowel wall thickness measurements were determined using a micrometer, and luminal-to-serosal distances were obtained from 10 points along the cross section. Colonic MPO activities were determined using the method of Yamada et al. (1993). Briefly, colon and spleen samples (10% w/v) were homogenized in 20 mM phosphate buffer (pH 7.4) and centrifuged at 6000 rpm for 20 min at 4°C. The pellets were homogenized and sonicated in 1 ml of 50 mM acetic acid (pH 6.0) containing 0.5% hexadecyltrimethylammonium hydroxide. MPO activities were determined using the H$_2$O$_2$-dependent oxidation of 3,3’,5,5’-tetramethylbenzidine and expressed as units/cm of colon or units/g of spleen. Plasma levels of nitrate and nitrite were determined as previously described using Aspergillus nitrate reductase to reduce all nitrate (NO$_3^-$) to nitrite (NO$_2^-$; Grassham et al., 1995). Colon and spleen samples from a second group of colitic rats ($n = 3$) and colitic rats treated with MG-341 ($n = 3$) were also obtained and frozen immediately in liquid nitrogen for subsequent Western blot analysis for iNOS protein.

**Western blot analysis.** The murine macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD), cultured in P-100 petri dishes, was preincubated in the presence or absence of 10 μM MG-341 contained within the media (Dulbecco’s modified Eagle’s medium) for 45 min. After stimulation with PG/PS (100 μg/ml) for 12 hr, cells were obtained by scraping, decanting the media and centrifuging at 1000 rpm for 5 min. The pellet was then resuspended in hot lysis buffer consisting of 125 mM Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, 0.2 mM phenylmethylsulfonyl fluoride and 1 μg/ml concentration each of pepstatin and leupeptin.

Colon and spleen samples were Dounce homogenized (20% w/v) in a protein extraction buffer consisting of 20 mM HEPES, pH 7.4, 0.1 mM EDTA, 12.5 mM MgCl$_2$, 150 mM NaCl, 0.1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol and 1 μg/ml concentration each of pepstatin, leupeptin and aprotinin. The homogenates were centrifuged at 12,000 g for 15 min.

**TABLE 1**

<table>
<thead>
<tr>
<th>Organ: Characteristic</th>
<th>Description</th>
<th>Score</th>
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<tbody>
<tr>
<td>Colon</td>
<td>Wall thickness</td>
<td>&lt;1 mm, transparent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~1 mm, mild visible thickening</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;1 mm, moderate to severe</td>
</tr>
<tr>
<td></td>
<td>Adhesions</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minimal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;3 isolated adhesions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Involving several bowel loops</td>
</tr>
<tr>
<td></td>
<td>Hyperemia</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Recognized</td>
</tr>
<tr>
<td>Spleen</td>
<td>Nodules</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small nodules, 0–50% coverage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large nodules, 50–100% coverage</td>
</tr>
<tr>
<td></td>
<td>Adhesions</td>
<td>None</td>
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<tr>
<td></td>
<td></td>
<td>0–50% attachment</td>
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<td></td>
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<td>50–100% attachment</td>
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</table>
genates were transferred to Eppendorf tubes, sonicated for 10 sec and then centrifuged at 14,000 rpm for 10 min.

Cell and tissue supernatants were removed and protein concentrations were determined using the modified Lowry DC Protein Assay (BioRad Laboratories, Hercules, CA). Cell (25 μg of protein) and tissue (100 μg of protein) supernatants were electrophoresed on 10% SDS-polyacrylamide gels at 100 V for 3 hr with prestained molecular weight markers (Amersham, Arlington Heights, IL). Proteins were then transferred onto 0.2-μm pore nitrocellulose membranes (Sigma, St. Louis, MO) in 25 mM Tris, 190 mM glycine, 0.1% SDS and 20% methanol. The membranes were blocked overnight at 4°C with 5% milk or 2% bovine serum albumin in phosphate-buffered saline. The membranes were then incubated for 1 hr at 4°C with monoclonal anti-mouse iNOS IgG (Transduction Labs., Lexington, KY) using a 1:1000 dilution; for RAW-derived samples, a polyclonal anti-rabbit iNOS antibody was used. After washing, the membranes were incubated for 2 hr with a 1:1000 dilution of anti-mouse (or anti-rabbit) IgG, F(ab')2 conjugated to alkaline phosphatase (Sigma). After washing, the membrane was developed in 50 ml of a chromogenic buffer solution containing 50 mM Tris, pH 9.5, 150 mM NaCl, 50 mM MgCl2, 0.1 mg/ml bromochloroindolyl phosphate and 0.2 mg/ml nitroblue tetrazolium.

**RNA extraction and Northern blot analysis.** RNA extraction was performed using a modification of Cathala *et al.* (1983). Colonic samples were immediately homogenized (20% w/v) in lysis buffer containing 5 M guanidine thiocyanate, 10 mM EDTA, 50 mM Tris and 8% β-mercaptoethanol and stored at −80°C. Thawed lysates were mixed with 4 M lithium chloride (1.7 w/v), left overnight at 4°C and then centrifuged 11,000 × g for 90 min at 4°C. RNA pellets were resuspended in elution buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.2% SDS), vortexed with an equal volume of Tris-buffered phenol and then centrifuged at 3000 rpm for 5 min. The aqueous phase was removed, and extraction was repeated three times with Tris-buffered phenol, Tris-buffered phenol/chloroform/isooxymal alcohol (25:24:1) and chloroform, respectively. The aqueous phase was removed; 0.1 volume of 4 M LiCl and 2.5 volumes of 100% ethanol were added, vortexed and inverted and then placed at −20°C for 2 hr. Precipitated RNA was obtained by centrifugation at 11,000 × g for 15 min at 4°C. RNA was resuspended in RNase-free water, and the quality and quantity were checked on a 1% agarose gel. Northern blot analysis was performed as previously described (Sambrook *et al.*, 1989). The rat VCAM-1 probe was a gift from Dr. Tucker Collins (Brigham and Women’s Hospital, Harvard University, Boston, MA).

**Statistical analyses.** Standard statistical methods were used. All data are expressed as the mean ± S.E.M. Statistical differences were identified using one-way analysis of variance, and multiple comparisons were performed using the Newman-Keuls *post hoc* analysis. Statistical significance was accepted at the .05 confidence interval.

**Results**

Chronic granulomatous colitis induced by the subserosal injection of PG/PS into the distal colon is characterized by colonic nodules, adhesions and thickening as well as an extensive mononuclear and polymorphonuclear cell infiltrate in the colonic interstitium (Aiko *et al.*, 1997; Grisham *et al.*, 1994, 1996; Yamada *et al.*, 1993). Corresponding with this chronic colitis is the dramatic up-regulation of iNOS in the colon (fig. 1). Daily oral administration of MG-341 significantly attenuated this enhanced expression of iNOS in the colon compared with tissue derived from vehicle-treated controls (fig. 1). This inhibition appears to be due to a direct attenuation of cellular iNOS expression rather than a non-specific secondary effect as MG-341 also inhibited iNOS protein expression in a murine macrophage cell line in vitro (fig. 2). In addition to inhibiting iNOS expression *in vivo*, we found that the proteasome inhibitor attenuated transcription of the endothelial cell adhesion molecule VCAM-1 in colonic samples (fig. 3). Coincident with this inhibition of gene transcription, we found that daily oral administration of MG-341 significantly inhibited the macroscopic signs of inflammation such that 0.3 mg/kg/day dose of the drug virtually eliminated bowel wall thickening, hyperemia and adhesions (table 2). Histological inspection of the colons revealed that PG/PS induced a marked increase in bowel wall thickness (fig. 4, A and B) and that animals treated with MG-341 exhibited an attenuation in bowel wall thickness compared with vehicle-treated colitic animals (fig. 4, B and C).

Quantitative histological analysis of these colons verified that treatment with the proteasome inhibitor significantly attenuated the increases in bowel wall thickness produced by intramural injection of PG/PS (table 2). In addition, MG-341 attenuated the PG/PS-induced increase in colonic dry weight/em as well (table 2). Using colonic MPO activity as a quantitative index of granulocyte infiltration (e.g., neutro...
phils, monocytes and eosinophils), we found that MG-341 significantly reduced PG/PS-induced increases in MPO activity (fig. 5). This inhibition in leukocyte infiltration was verified by histological inspection of the tissue (fig. 4, E and F).

As previously reported, the intramural injection of PG/PS into the distal colon not only produces colitis but also induces a chronic granulomatous inflammation of the spleen as seen grossly by the appearance of splenic nodules and adhesions (Aiko et al., 1997; Grisham et al., 1994, 1996; Yamada et al., 1993). This chronic inflammatory response in the spleen also corresponded to a significant and sustained up-regulation of iNOS in the spleen, which was inhibited by daily administration of MG-341 (fig. 1). Furthermore, inhibition of expression of both spleen and colon iNOS also resulted in a significant inhibition of plasma levels of NO$_3^-$ and NO$_2^-$, the metabolic products of NO (fig. 6). The increases in splenic macroscopic inflammation and spleen weight induced by PG/PS were significantly reduced by daily administration of MG-341 (table 2). Histological inspection of spleens showed an attenuation of PG/PS-induced increase in inflammatory cells and a decrease in the appearance of fibroblasts and deposition of connective tissue (fig. 7). In addition, MG-341 significantly attenuated the PG/PS-induced increases in splenic MPO activity (fig. 5).

Discussion

There is a growing body of both experimental and clinical data demonstrating that chronic gut inflammation is associated with enhanced production of NO (Aiko and Grisham, 1995; Berg et al., 1996; Boughton-Smith et al., 1993; Grisham et al., 1994; Hogaboam et al., 1995; Middleton et al., 1993; Miller et al., 1992; Rachmilewitz et al., 1995; Ribbons et al., 1995; Yamada et al., 1993). Evidence is accumulating to support the idea that the large amounts of NO generated by iNOS may be proinflammatory and are likely to be involved in modulating acute and chronic inflammation. Indeed, previous studies from our laboratory have shown that chronic iNOS inhibition using aminoguanidine, a relatively selective inhibitor of iNOS, significantly attenuates the chronic colitis induced by PG/PS or the spontaneous colitis observed in HLA-B27 transgenic rats (Aiko and Grisham, 1996; Grisham et al., 1994). Although iNOS expression has been demonstrated to involve the proteasome-dependent activation of NF-$\kappa$B (Griscavage et al., 1996), there has been no attempt to determine whether the 26S proteasome is involved in the regulation of expression of iNOS in chronic inflammation in vivo. We found that daily administration of a new orally active proteasome inhibitor significantly inhibited the PG/PS-induced up-regulation of iNOS in the colon and spleen (fig. 1) and attenuation in plasma levels of NO$_2^-$ and NO$_3^-$ in colitic rats (fig. 6). Furthermore, we found that MG-341 inhibited the PG/PS-induced up-regulation of iNOS in vivo in a macrophage cell line (fig. 2), suggesting that proteasome inhibition did indeed directly inhibit expression of active iNOS protein.

The cellular sources of this iNOS are not known with certainty, but likely candidates would include the phagocytic leukocytes such as neutrophils, monocytes and macrophages. This hypothesis is supported by data demonstrating that PG/PS enhances granulocyte infiltration into the colon and spleen, which correlates well with the increase in iNOS expression (figs. 4, 5 and 7). In many respects, these data are very similar to those obtained in previous studies using the relatively selective inhibitor of iNOS (i.e., aminoguanidine) in which we demonstrated significant anti-inflammatory activity in this model of colitis (Aiko and Grisham, 1996; Grisham et al., 1994). In the present study, we show that a selective proteasome inhibitor also attenuates the chronic inflammation in both the colon and spleen as measured by attenuation in tissue MPO activity, organ weight and histological inflammation (figs. 4, 5 and 7, table 2). The mechanisms by which the sustained overproduction of NO may promote inflammation are undefined; however, there are several possibilities. For example, it has been demonstrated that NO or NO-derived metabolites may promote leukocyte chemotaxis (Beauvais et al., 1995). NO has also been shown to mediate cellular injury and/or apoptosis (Brune et al., 1995; Fukuo et al., 1996; Shimaoka et al., 1995). Finally, NO may promote inflammation by enhancing the production of proinflammatory cytokines such as IL-8 (Remick and Villarete, 1996).

Although it is tempting to speculate that the inhibition of iNOS expression by oral administration of a proteasome inhibitor is a major pathway by which chronic inflammation is attenuated, it should be pointed out that the proteasome pathway plays an important role in several other physiological processes. It is known, for example, that the proteasome pathway is the major pathway by which damaged or mutated proteins are degraded by the cell (Goldberg, 1992). More recent studies suggest that the 26S proteasome complex may also be involved in the regulation of other cellular functions, such as proliferation, antigen presentation and transcription factor regulation (Ciechanover, 1994; Goldberg and Rock, 1992). Studies by King et al. (1995) and Yaglom et al. (1995) have demonstrated that the ubiquitination-proteasome-
mediated degradation of certain cyclins is necessary for controlling cell growth and metabolism. In addition, recent work by Rock et al. (1994) has shown that the proteasome is essential for generation of most of the peptides presented on MHC class I molecules. Finally, the ubiquitin-proteasome pathway has been shown to regulate the activation of NF-κB (Baeuerle and Henkle, 1994; Palombella et al., 1994).

The proteasome appears to play an important role in the regulation of inflammation through its degradation of IκBα, the inhibitory factor for the transcription factor NF-κB. Limited proteolysis of the inactive NF-κB/IκBα complex generates the active form of NF-κB, which translocates into the nucleus and initiates the transcription of a wide variety of NF-κB-responsive, proinflammatory genes. Studies in vitro have shown a wide range of proinflammatory stimuli such as

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TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Colonic macroscopic score</th>
<th>Bowel wall thickness μm</th>
<th>Colonic dry weight mg dry wt/cm</th>
<th>Spleen macroscopic score</th>
<th>Spleen weight mg/g b.wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + 0.5% methylcellulose</td>
<td>0 ± 0</td>
<td>680 ± 10.3</td>
<td>0.015 ± 0.0006</td>
<td>0 ± 0</td>
<td>2.77 ± 0.086</td>
</tr>
<tr>
<td>Control + 0.3 mg/kg MG-341</td>
<td>0 ± 0</td>
<td>832 ± 44.7</td>
<td>0.017 ± 0.0013</td>
<td>0 ± 0</td>
<td>2.36 ± 0.130</td>
</tr>
<tr>
<td>PG/PS + 0.5% methylcellulose</td>
<td>3.3 ± 0.28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1202 ± 39.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.032 ± 0.0025&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.3 ± 0.28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.54 ± 1.092&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PG/PS + 0.3 mg/kg MG-341</td>
<td>0 ± 0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>949 ± 33.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.023 ± 0.0009&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.83 ± 0.123&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> Bowel wall thickness was obtained by viewing 2-μm transverse sections of colon stained with H&E at ×20 and ×100, measuring wall thickness from lumen to serosa using a micrometer. Measurements were taken from 10 points around the cross section.

<sup>b</sup> Colons were excised, and wet weight and length were determined. A transverse sample was obtained, weighed, dried for 48 hr at 80°C and then reweighed. Values are expressed as mg dry weight/cm of colon.

<sup>c</sup> P<sup>b</sup>.05 and <sup>d</sup> P<sup>b</sup>.01 compared with controls, <sup>e</sup> P<sup>b</sup>.01 compared with the vehicle-treated PG/PS group.

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Fig. 4. Effect of proteasome inhibition on PG/PS-induced histopathology of the colon 2 weeks after the induction of colitis. A, Saline control; the colon exhibits normal mucosal and submucosal morphology and bowel wall thickness. B, PG/PS and vehicle-treated animals; the colon exhibits dramatic thickening of the submucosa along with loss of normal mucosal convolutions and significant inflammatory cell infiltration into the submucosa. C, PG/PS and 0.3 mg/kg/day MG-341-treated animals; the bowel wall appears relatively normal with respect to mucosal and submucosal morphology. The extensive inflammatory cell infiltrate has been reduced. Magnification for A–C, 40×. D, Saline control; normal morphology and relationship of the mucosa, submucosa and muscularis externa. E, PG/PS and vehicle-treated animals; the submucosa is increased in thickness and contains an extensive inflammatory cell infiltrate composed primarily of mononuclear cells and a few PMNs. F, PG/PS and MG-341-treated animals; the infiltration of inflammatory cells into the submucosa is reduced and the thickness of this region is diminished. Magnification for D–F, 100×.

Fig. 5. Effect of proteasome inhibition on PG/PS-induced increases in colonic (top) and splenic (bottom) MPO activity. MG-341 was administered orally at a dose of 0.3 mg/kg/day for 14 days. Colonic MPO was determined, at 2 weeks after colitis induction, using the H<sub>2</sub>O<sub>2</sub>-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine. Each bar represents mean ± S.E.M. * P<sup>b</sup>.05 compared with controls, §P<sup>b</sup>.05 compared with vehicle-treated PG/PS rats.
tumor necrosis factor-α (Osborn et al., 1989), interleukin-1 (Iwasaki et al., 1992), hydrogen peroxide (Schreck et al., 1992) and lipopolysaccharide (Muller et al., 1993) to activate NF-κB. NF-κB activation stimulates the production of various proinflammatory and chemotactic agents such as the interleukins-1, -2, -6 and -8 (Cogswell et al., 1994; Hoyos et al., 1989; Kunsch and Rosen, 1993; Lieberman and Baltimore, 1990; Mukaida et al., 1994), tumor necrosis factor-α (Shakhov et al., 1990), iNOS (Adcock et al., 1994) and various adhesion molecules. Induction of endothelial cell-surface expression of leukocyte adhesion molecules E-selectin, VCAM-1 and ICAM-1 by tumor necrosis factor-α, when blocked by selective proteasome inhibition, results in reduced leukocyte rolling/adhesion and transmigration (Read et al., 1995). In view of the proinflammatory processes stimulated as a consequence of NF-κB activation, coupled to the fact that the proteasome may occupy an important position in this process, a second objective of this study was to assess the anti-inflammatory properties of the new proteasome inhibitor in the PG/PS model of chronic colitis.

We found that daily oral dosing with MG-341 attenuated colonic and splenic inflammation as judged by dramatic reductions in their macroscopic inflammatory scores (table 2). In addition, we found that MG-341 attenuated increases in leukocyte infiltration into the colon and spleen (figs. 4, 5 and 7). One potential mechanism whereby proteasome inhibition attenuates leukocyte recruitment into the interstitium is by inhibition of endothelial cell expression of ICAM-1 and VCAM-1.

Endothelial cell VCAM-1 interacts with VLA-4 expressed on lymphocytes, monocytes and eosinophils (Chan et al., 1992; Elices et al., 1990). During chronic inflammation, the endothelial expression of VCAM-1 mediates lymphocyte, monocyte and eosinophil recruitment to the sites of inflammation. This observation may be particularly important in our model of colitis because we have shown that T lymphocytes are essential for full expression of the chronic colitis produced by PG/PS (Aiko et al., 1997). We have demonstrated that the immunosuppressive agents cyclosporin A and FK506 possess potent anti-inflammatory activity in our model of colitis (Aiko et al., 1997). Data obtained in the present study demonstrate that MG-341 effectively inhibits VCAM-1 expression in the inflamed colon (fig. 3). Attenuating the transcription and subsequent surface expression of VCAM-1, through proteasome inhibition, may suppress the infiltration of lymphocytes to the inflammatory site. An interesting, yet perplexing observation made in this model of colitis is that although NO has been proposed to downregulate endothelial cell expression of VCAM-1 in vitro (De Caterina et al., 1995; Khan et al., 1996), we observed massive mononuclear leukocyte infiltration and VCAM-1 upregulation in the presence of large amounts of iNOS-derived NO. These data suggest that the acute effect of NO on endothelial cell adhesion molecule expression in vitro may be much more complex in in vivo models of chronic inflammation.

Another striking observation made during the course of these studies was the remarkable attenuation of the PG/PS-induced colonic and splenic adhesions by the proteasome inhibitor (table 2). The mechanisms by which MG-341 attenuate adhesions between tissues or between the tissues and the abdominal wall remain only speculative but may involve...
inhibition of fibrosis by the proteasome inhibitor. Indeed, MG-341 appears to inhibit colonic fibrosis as judged by its ability to reduce PG/PS-dependent increases in bowel wall thickness and dry weight (table 2). These PG/PS-induced increases in bowel wall thickness and weight were not due to edema because there were no significant differences in the colonic wet-to-dry ratios between groups (data not shown). In Crohn’s disease, submucosal inflammation is a prerequisite for collagen deposition and fibrosis (Graham et al., 1988). Inflammatory cells in the submucosa can regulate the activation and proliferation of fibroblasts and/or smooth muscle cells through the release of fibrogenic cytokines and growth factors such as platelet-derived growth factor, interleukin-1, transforming growth factor-β and fibroblast growth factor (Freundlich et al., 1986; Gospodarowicz et al., 1986; Grotendorst, 1988; Postlewaite et al., 1987). PG/PS-induced colitis is characteristic of a fibrotic submucosal inflammation, and therefore the ability of MG-341 to diminish fibrosis may be a consequence of its ability to inhibit leukocyte infiltration as well as direct inhibition of fibroblast and smooth muscle collagen deposition. Additional studies will be required to determine the specific mechanisms.

In summary, our data demonstrate that inhibiting the 26S proteasome attenuates both the chronic gut and spleen inflammation induced by PG/PS. The mechanisms by which this inhibition down-regulates the inflammatory response in this model of colitis include inhibition of expression of iNOS, adhesion molecules and/or proinflammatory cytokines or mediators. Inhibiting the inflammatory process may also attenuate a number of “secondary” features of chronic inflammation—such as tissue adhesions and fibrosis. Taken together, these results indicate that the 26S proteasome occupies an important position in the inflammatory cascade and may represent a potential target for down-regulating the inflammatory response.

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Send reprint requests to: Elaine M. Conner, Ph.D., Department of Molecular and Cellular Physiology, Louisiana State University Medical Center, P.O. Box 33932, 1501 Kings Highway, Shreveport, LA 71130.