Effects of Nitric Oxide Synthase Inhibition or Sulfasalazine on the Spontaneous Colitis Observed in HLA-B27 Transgenic Rats

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Abstract

The objective of this study was to determine the effects that certain nitric oxide synthase inhibitors have on the spontaneous intestinal and colonic inflammation that develops in HLA-B27 transgenic rats and compare these data to those obtained using sulfasalazine (SZ). In an attempt to more closely mimic the clinical situation, drug treatment was begun after the onset of colitis. HLA-B27 male rats that developed clinical signs of colitis (diarrhea/loose stools) at 17 wk of age were randomized into four groups consisting of one untreated colitic group and three treatment groups that received either aminoguanidine (AG; 52 μmol/kg/day), N^G^-nitro-L-arginine methyl ester (L-NAME; 45 μmol/kg/day) or SZ (130 mg/kg/day) in their drinking water for 14 days. Aged-matched Fisher 344 male rats were used as healthy controls. After 3 wk of treatment, ileal and colonic mucosal permeabilities, granulocyte infiltration and nitric oxide were quantified using blood-to-lumen clearance of ^51^Cr-EDTA, tissue myeloperoxidase activity, and plasma levels of nitrate and nitrite, respectively. We found that both AG and L-NAME but not SZ significantly attenuated the increases in plasma nitrate and nitrite levels. Interestingly, all three drugs were effective at significantly attenuating the increases in myeloperoxidase activity in the distal colon. Treatment with AG and SZ but not L-NAME were effective at significantly attenuating the increase in ileal and colonic permeabilities. Quantitative histological analysis revealed that AG and L-NAME but not SZ significantly attenuated the increase in the mucosal thickness and crypt depth in the distal colon compared to untreated colitics. Taken together, these data demonstrate that oral administration of certain nitric oxide synthase inhibitors or SZ to animals with active colitis attenuates the colonic inflammation by at least two different mechanisms. One mechanism appears to be dependent on inhibition of NO production whereas the other mechanism does not.

It is becoming increasingly apparent that chronic intestinal and/or colonic inflammation observed in experimental models of IBD or in human IBD is associated with the sustained overproduction of NO (Miller et al., 1992; Yamada et al., 1993; Middleton et al., 1993; Boughton-Smith et al., 1993; Grisham et al., 1994; Lundberg et al., 1994; Aiko and Grisham, 1995; Hogaboam et al., 1995; Rachmilewitz et al., 1995). As yet, the question of whether this sustained elevation of NO production is a cause or consequence of chronic gut inflammation remains to be answered. We, as well as others, have found that certain NOS inhibitors attenuates the intestinal and colonic inflammation induced in rodents by a variety of different agents (Miller et al., 1992; Grisham et al., 1994; Aiko and Grisham, 1995; Hogaboam et al., 1995). In a model of PG/PS-induced chronic granulomatous colitis we found that AG and to a lesser extent L-NAME significantly inhibited granuloma formation and colonic inflammation at concentrations that were determined to be ineffective at altering blood flow in the normal rat gastrointestinal circulation (Grisham et al., 1994). It should be noted that virtually all published studies to date using NOS inhibitors have been performed using a pretreatment protocol rather than a therapeutic protocol. This has prompted some investigators to question whether the timing of administration of the NOS inhibitor is important in producing its antiinflammatory effect (Laszlo et al., 1994). We have found that SZ is also effective at attenuating the PG/PS-induced granulomatous colitis when administered immediately after induction of colitis suggesting the possibility that SZ may directly or indirectly mediate its antiinflammatory activity by attenuating NO production (Grisham et al., 1996). Recent studies from our laboratory have demonstrated that the onset of the spontaneous ileal and colonic inflammation that develops in HLA-
B27 transgenic rats corresponds with a significant increase in NO metabolism (Aiko and Grisham, 1995). Because no studies have been performed using NOS inhibitors or SZ therapeutically in spontaneous models of colitis or ileitis, we wished to determine what effect certain NOS inhibitors or SZ have on the spontaneous intestinal and colonic inflammation that develops in the HLA-B27 transgenic rats. In an attempt to more closely mimic the clinical situation, drug treatment was begun after the onset of colitis.

Materials and Methods

Animals. HLA-B27 transgenic male rats (derived from Fisher 344 rats; n = 28) were obtained from GenPharm International (Mountain View, CA) at 9 wk of age (weight range, 138–220 g). The animals were housed under specific pathogen-free conditions in wire-mesh bottom cages, and were given free access to water and standard laboratory rat chow. Animals were observed for clinical symptoms of colitis (loose stools and/or diarrhea). Body weights of each animal were measured and recorded at 7-day intervals. At 20 wk of age, the two HLA-B27 transgenic rats that did not exhibit the symptoms of colitis were excluded from this study. Previous work from our laboratory has demonstrated a direct correlation between loose stools/diarrhea and histopathological and biochemical evidence of active colitis (Aiko and Grisham, 1995). Consequently, a total of 26 transgenic rats were entered into this study and were randomized into one untreated group (n = 7) and three treatment groups which were treated with either AG (n = 7, hemisulfate salt), L-NAME (n = 7) or SZ (n = 5). All drugs were administered to the transgenic animals in their drinking water for 3 wk. The volume of drinking water that each rat consumed for a given period was measured and recorded daily. We found that the rats consumed 174 ± 9, 150 ± 9, and 131 ± 3 ml/kg/day of AG, L-NAME and sulfasalazine, respectively. This represented doses of 52 and 45 μmol/kg/day for AG and L-NAME, respectively, and 130 mg/kg/day sulfasalazine. Fisher 344 rats used as a healthy control group (n = 8) and were obtained from Tacomics Inc. (Germantown, NY) at 20 wk of age and housed for an additional 3 wk as described above. After 3 wk of treatment with AG, L-NAME or sulfasalazine, all control and transgenic rats were fasted for 24 hr.

Surgery and mucosal permeability measurements. After the 24-hr fast, all rats were weighed and anesthetized with an i.p. injection of 120 mg/kg sodium 5-ethyl-1 (1-methyl-propyl)-2-thiobarbiturate (Inactin, Byk-Gulden, Konstanz, Germany). Body temperature was maintained at 37°C with a thermost-controlled water pad (Aquamatic K-Modules K-20; Baxter, Valencia, CA). The animals underwent tracheostomy, and the right femoral artery was cannulated for arterial pressure recording and blood sampling. The right femoral vein was also cannulated for injection of the isotope marker. A laparotomy was performed using a midline abdominal incision. Both renal vessels were ligated to prevent rapid excretion of the radioisotope marker into the urine. The ileum was cannulated at both 10 and 3 cm proximal to the cecum using Silastic tubing (Dow Corning, Arlington, TN; inner diameter 0.025 mm) and Silastic tubing (inner diameter, 0.25 mm) for infusion and collection of the modified Tyrodes’ solution, respectively. The descending colon was isolated and cannulated at both the splenic flexure and the rectum using the same type of tube. The perfused ileum and colon were returned to the abdominal cavity, and the abdominal wall was closed to minimize dehydration of the organs during the experiment. The luminal content of the ileum and colon was removed by perfusion of warm (37°C) modified Tyrode’s solution for 30 min.

Mucosal permeability was determined using the blood-to-lumen clearance of 51Cr-labeled EDTA as described previously (Aiko and Grisham, 1995). One hundred microcuries of 51Cr-EDTA (Du Pont de Nemours & Co., Boston, MA) was injected via the femoral vein catheter. After a 15-min equilibration period, the perfusate was collected every 10 min for 40 min from each catheter individually for the appearance of 51Cr-EDTA. Plasma samples were obtained at 40 min for use as reference counts and assessment of circulating nitrate and nitrite levels. The urine was also obtained directly from the bladder for the determination of urinary nitrate and nitrite levels. Blood-to-lumen clearance of 51Cr-EDTA was calculated using the formula described previously.

Tissue preparation and biochemical analysis. After the determinations of mucosal permeability, the animals were euthanized with an overdose of pentobarbital sodium (Butler, Columbus, OH) and the perfused ileum and distal colon were excised. The proximal colon were also excised and the luminal contents were removed with saline. The ileum and colon were opened longitudinally. The length and weight of each organ was recorded, and each tissue was sectioned for histological analysis, wet-to-dry measurements, and MPO determinations. Wet-to-dry weight ratios were calculated by dividing the wet weight of each sample by its dry weight prepared following a 48-hr incubation at 80°C. MPO activity was determined as described previously (Aiko and Grisham, 1995). MPO activity was expressed as units per centimeter of ileum or colon.

Plasma levels of nitrate and nitrite were determined spectrophotometrically using Aspergillus nitrate reductase and the Griess reagent (1% sulfanilamide, 1% naphthylenediamine dihydrochloride and 2.5% H3PO4) as described previously (Grisham et al., 1996). For histological analysis, tissue samples (ileum, proximal and distal colon) were obtained from each animal, fixed, dehydrated and embedded in JB-4 (Polysciences, Inc., Warrington, PA). Sections (2–3 μm) were cut with glass knives and stained with H&E. Bowel wall thickness, mucosal thickness, submucosal thickness and crypt depth were quantified from 5 high powered fields from at least two different sections from each specimen.

Statistical analyses. All results are expressed as mean ± S.E.M. The multiple comparisons were performed using Fisher’s PLSD. Results were considered statistically significant at a P value of <.05.

Results

We found that only L-NAME administration adversely affected body weight during the 3-wk treatment period. In fact, these animals lost approximately 15% of their body weight following L-NAME treatment (283 ± 9 vs. 241 ± 8 g). We also found that only AG treatment but not L-NAME or SZ reduced the incidence of loose stools/diarrhea from 100 to 57% beginning the second wk of treatment (data not shown). Indeed, approximately 14% of rats treated with L-NAME developed diarrhea with a positive occult blood reaction after 3 wk of treatment (data not shown).

Heart rates and mean arterial blood pressures were also recorded in all rats after 3 wk of treatment. Heart rates were significantly decreased in all three treatment groups when compared to the untreated colitic group (354 ± 12 vs. 303 ± 6, 264 ± 15 and 312 ± 17 bpm for untreated colitis vs. AG, L-NAME and SZ treated, respectively). Mean arterial blood pressure was significantly increased only in the rats treated with L-NAME when compared to the untreated colitic group (133 ± 2 vs. 209 ± 5 mm Hg for untreated colitis vs. L-NAME treated).

Figure 1 shows the effects of AG, L-NAME or SZ treatment on granulocyte infiltration as measured by MPO activity in the ileum, proximal and distal colon. We found that the increase in ileal MPO activity was not significantly reduced by any treatment, whereas AG and L-NAME significantly attenuated the MPO activity in the proximal colon. Interestingly, AG, L-NAME and SZ all significantly attenuated the rise in MPO activity in the distal colon (fig. 1).

AG and SZ were also found to significantly attenuate ileal

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and colonic mucosal permeabilities when compared to the untreated colitic group (fig. 2). In contrast, administration of L-NAME did attenuate the ileal mucosal permeability and in fact actually enhanced colonic permeability compared to the untreated colitic group (fig. 2). Another characteristic of this model of colitis is the increase in dry weight per unit length of the colon. We found that AG and SZ actually increased dry weight of the ileum when compared to the untreated colitis (table 1). Dry weights of the proximal and distal colon in the untreated group were significantly enhanced compared to the control and neither AG, L-NAME nor SZ attenuated this increase (table 1). Wet-to-dry ratios in the ileum were also significantly increased when compared to controls and all the drugs significantly attenuated this increase (table 2). Although the wet-to-dry ratio of the proximal colon was increased in the untreated colitic group, none of three drugs were effective at attenuating this increase (table 2). Interestingly, active distal colitis was not associated with an increase in water content in the tissue (table 2). In fact, all three drugs significantly reduced the wet-to-dry ratios indicating loss of interstitial fluid accumulation (table 2). Histological inspection of the colon revealed an increase in the bowel thickness with an extensive inflammatory cell infiltration and hyperplasia of crypt epithelial cells in the untreated HLA-B27 rats when compared to their Fisher 344 controls (fig. 3). The thickness of the mucosa appeared to be attenuated in AG and L-NAME groups compared to the untreated group. Quantitative histological analysis showed that both AG and L-NAME significantly attenuated the mucosal thickness and crypt depth in the distal colon whereas only L-NAME enhanced submucosal thickness (fig. 4). Plasma levels of nitrate and nitrite, the stable auto-oxidation products of NO were also significantly decreased in the rats treated with both AG and L-NAME, whereas SZ had no affect on plasma levels of these nitrogen oxides (fig. 5).

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ileum</th>
<th>Proximal Colon</th>
<th>Distal Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.008 ± 0.0006</td>
<td>0.011 ± 0.0006</td>
<td>0.017 ± 0.001</td>
</tr>
<tr>
<td>Untreated colitics</td>
<td>0.009 ± 0.0005</td>
<td>0.019 ± 0.0002</td>
<td>0.026 ± 0.001</td>
</tr>
<tr>
<td>AG-treated</td>
<td>0.012 ± 0.0009a</td>
<td>0.019 ± 0.0001</td>
<td>0.027 ± 0.0009</td>
</tr>
<tr>
<td>L-NAME-treated</td>
<td>0.011 ± 0.0008a</td>
<td>0.018 ± 0.0008</td>
<td>0.026 ± 0.0002</td>
</tr>
<tr>
<td>SZ-treated</td>
<td>0.013 ± 0.0001a</td>
<td>0.022 ± 0.0009a</td>
<td>0.031 ± 0.0001a</td>
</tr>
</tbody>
</table>

All data are expressed as the mean ± S.E.M. *P < .05 compared to controls.

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ileum</th>
<th>Proximal Colon</th>
<th>Distal Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.1 ± 0.13</td>
<td>3.9 ± 0.2</td>
<td>5.8 ± 0.16</td>
</tr>
<tr>
<td>Untreated colitics</td>
<td>4.7 ± 0.11a</td>
<td>4.6 ± 0.14a</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>AG-treated</td>
<td>3.7 ± 0.26</td>
<td>4.3 ± 0.19</td>
<td>4.7 ± 0.13a</td>
</tr>
<tr>
<td>L-NAME-treated</td>
<td>4.1 ± 0.1</td>
<td>5.0 ± 0.12a</td>
<td>4.8 ± 0.07b</td>
</tr>
<tr>
<td>SZ-treated</td>
<td>4.2 ± 0.2</td>
<td>4.4 ± 0.2</td>
<td>4.6 ± 0.15a</td>
</tr>
</tbody>
</table>

All data are expressed as the mean ± S.E.M. *P < .05 compared to controls. 

Fig. 1. MPO activities in the ileum, proximal colon and distal colon in healthy Fisher 344 rats (control), untreated HLA-B27 rats, and HLA-B27 rats treated with AG, L-NAME or sulfasalazine. Rats were treated for 3 wk after the onset of colitis. *P < .05 compared to healthy controls.

Fig. 2. Mucosal permeabilities of the ileum and colon in Fisher 344 controls, untreated HLA-B27 rats and HLA-B27 rats treated with AG, L-NAME or sulfasalazine. Rats were treated for 3 wk after the onset of colitis. *P < .05 compared to healthy controls and 1P < .05 compared to untreated colitic group.
Discussion

One of the most consistent findings using a variety of different animal models of IBD is that NOS inhibitors appear to attenuate intestinal and/or colonic inflammation (Miller et al., 1992; Grisham et al., 1994; Aiko and Grisham, 1995; Hogaboam et al., 1995). Virtually all of these studies have used induced models of intestinal and/or colonic inflammation and some form of a pretreatment protocol, i.e., the NOS inhibitors were administered either before or immediately after induction of colitis. This has raised the question of whether or not NOS inhibitors would be as effective if administered therapeutically once the inflammation has occurred and whether NOS inhibitors would be as effective in genetically engineered models of gut inflammation. Thus, a major objective of our study was to assess the antiinflammatory properties of two different NOS inhibitors in a spontaneous model of chronic colitis administered after the onset of colitis. A second objective was to compare these data to those obtained using SZ, a drug known to be effective in the treatment of human UC. Aminoguanidine and L-NAME were chosen as the NOS inhibitors for two reasons. First, AG has been shown to be more selective in its ability to inhibit iNOS, whereas L-NAME is known to be more selective toward the constitutive (i.e., endothelial and neuronal) NOS. Second, AG and L-NAME have been found to possess varying degrees of anti-inflammatory activity in a model of chronic granulomatous colitis (Grisham et al., 1994).

We found that a 3-wk treatment protocol with either L-NAME, AG or SZ were effective at inhibiting granulocyte infiltration into the distal colon as measured by attenuation in colonic MPO activity (fig. 1). This anti-inflammatory activity was confirmed, using histologic inspection of the tissue (fig. 3). Interestingly, both L-NAME and AG were effective at inhibiting NO production as measured by their ability to attenuate the rise in plasma levels of nitrate and nitrite, whereas SZ did not (fig. 5). Furthermore, SZ was not effective at inhibiting granulocyte accumulation in the proximal colon (fig. 1). This may be due to location of specific enteric bacteria necessary to reduce the azo bond of SZ, thereby liberating the active anti-inflammatory moiety of SZ, 5ASA. Taken together, these data suggest that the granulocyte recruitment into the distal colon in this model of spontaneous colitis may be dependent on NO-dependent and -independent pathways. These data contrast with those reported by Ribbons et al. (1997) in which selective inhibitors of iNOS were administered to rhesus monkeys with established colitis. The authors found no significant anti-inflammatory effect. The reasons for these apparent discrepant results are not clear at the present time, however, it may be that the metabolism of the iNOS inhibitors is very different in rats vs. monkeys.
The mechanisms by which NOS inhibitors attenuate leukocyte infiltration have not been clearly delineated; however, there are several possibilities. For example, it has been demonstrated that NO or NO-derived metabolites may promote chemotaxis of some leukocytes in vitro (Kaplan et al., 1989; Beauvais et al., 1995). Another mechanisms suggests that NO may directly or indirectly mediate epithelial cell toxicity and/or apoptosis thereby releasing proinflammatory mediators (Sandoval et al., 1995; Tepperman et al., 1993; Xie et al., 1993; Mebmer et al., 1995). This possibility may not be as important in the HLA-B27 model of colitis based upon our findings that although L-NAME inhibits NO production, it did not attenuate colonic mucosal injury (see below; fig. 2). Indeed, constitutive NOS may be important for maintaining barrier function (Kubes, 1993; Miller et al., 1993). NO or NO-derived metabolites may promote leukocyte infiltration in an indirect manner by enhancing the production of proinflammatory mediators such as IL-8 or TNF (Villarete et al., 1995; Mebmer et al., 1995). Furthermore, Lander and coworkers have demonstrated that NO or one of its auto-oxidation products activates lymphocytes to produce TNF (Lander et al., 1996). Finally, the vasoactive properties of certain NOS inhibitors may contribute to their anti-inflammatory activity. For example, it is possible that inhibition of endothelial NOS may protect the gut by promoting vasoconstriction which would decrease blood flow thereby limiting the delivery of inflammatory cells and mediators to the tissue. However, we have been unable to observe any significant reductions in small intestinal and/or colonic blood flow in healthy rats when L-NAME or AG is administered orally for 21 days at the doses used in this and other studies (Aiko et al., 1995). Although AG has been shown to be a more selective inhibitor of iNOS than is L-NAME (Corbett et al., 1992; Griffiths et al., 1993; Cross et al., 1994; Wolff and Lubeskie, 1995; Ruetten and Thiemermann, 1996), it should be noted that AG is also known to inhibit histaminase (diamine oxidase) activity (Lindell et al., 1960), glycation of proteins (Brownlee et al., 1986), aldose reductase activity (Kumari et al., 1991), and oxidative modification of LDL (Picard et al., 1992). How these mechanisms would reduce colonic inflammation is not apparent but these alternative mechanisms should be considered.

The antiinflammatory of SZ may be of interest to those investigators who wish to use the HLA-B27 transgenic rat as a model of human IBD. The mechanisms by which SZ inhibit the recruitment of leukocytes into the distal colon again, remain only speculative. Sulphasalazine has been used for more than 40 yr to treat human distal bowel disease and yet there is no consensus as to the mechanisms by which this drug mediates its anti-inflammatory activity. Indeed, the idea that SZ or its active metabolite 5-ASA mediates its anti-inflammatory activity by acting as a 5-lipoxygenase inhibitor is not likely in view of the largely disappointing clinical studies using very selective inhibitors of 5-lipoxygenase activity. An alternative explanation may be the potent antioxidant properties of 5-ASA (Miles and Grisham, 1994). We, as well as others, have demonstrated that 5-ASA is capable of scavenging O$_2^-$, organic radicals and neutrophil-derived hypochlorous acid (Miles and Grisham, 1994). Antioxidants are known to attenuate the tissue injury and dysfunction in different animal models of gastrointestinal inflammation (reviewed in Granger et al., 1994). It is also intriguing to speculate that 5-ASA, by virtue of its potent antioxidant properties, may inhibit activation of certain transcription factors (i.e., NFkB) that are required for expression of pro-inflammatory adhesion molecules, cytokines and enzymes (Schreck et al., 1991; Schmidt et al., 1995; Sen et al., 1996). A recent preliminary report also suggests that 5-ASA may actually enhance the transcription of MnSOD within the gut epithelium which in turn may inhibit leukocyte infiltration (Valentine et al., 1995). An interesting observation made in our study was the ability of SZ to enhance mucosal thickness compared to untreated colitic rats (fig. 4). The reasons for this apparent hypertrophic effect are not clear at the present time but are in need of investigation.

Another interesting finding in this study was the fact that although L-NAME did attenuate leukocyte infiltration into the colon it did not reduce (and in some cases actually enhanced) mucosal injury. Indeed, L-NAME appeared to worsen the mucosal injury observed in these transgenic rats as witnessed by the larger amounts of occult blood in stool samples (data not shown) and enhanced permeability (fig. 2). Furthermore, L-NAME-treated animals lost significantly more body weight when compared to the other drug-treated or untreated animals (data not shown). Taken together, these data suggest that the sustained overproduction of NO is not responsible for the epithelial cell injury observed in this model of colitis. The mechanisms responsible for these unexpected results have not been identified but a reasonable assumption may be that chronic L-NAME administration inhibits endothelial and/or neuronal NOS which may enhance vascular permeability and epithelial cell injury. The ability of L-NAME to enhance vascular and mucosal permeability has been well documented in vivo (Kubes, 1993).

The results, however, contrast with data obtained in other models of induced colitis in which L-NAME was found to be protective (Miller et al., 1992; Grisham et al., 1994; Hogaboom et al., 1995; Rachmilewitz et al., 1995). For example, no such exacerbatory activity of L-NAME has been reported for acetic acid or TNBS-induced colitis or ileitis or in the chronic granulomatous colitis (Miller et al., 1992; Yamada et al., 1993; Grisham et al., 1994; Aiko and Grisham, 1995; Hogaboom et al., 1995; Rachmilewitz et al., 1995). These differences may represent differences in the pathogenesis of HLA-B27 transgenic rats vs. chemically or immunologically induced models of colitis or the pretreatment vs. therapeutic treatment protocols.

In summary, our data demonstrate that although therapeutic administration of AG, L-NAME or SZ to transgenic rats with established colitis significantly attenuates leukocyte infiltration into the distal colon, only AG and L-NAME significantly reduces the rise in plasma levels of NO-derived NO$_2^-$ and NO$_3^-$. Furthermore, only AG and SZ and not L-NAME attenuates mucosal barrier dysfunction suggesting that NO-dependent and -independent pathways promote leukocyte infiltration and tissue dysfunction in this model of spontaneous colitis.

References


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