DUAL ROLE OF ENDOGENOUS NITRIC OXIDE IN DEVELOPMENT OF DEXTRAN SULFATE SODIUM-INDUCED COLITIS IN RATS

The role of nitric oxide (NO) in the etiology of ulcerative colitis is controversial with reports of the improvement and aggravation of colonic lesions by inducible NO synthase (iNOS) inhibitors. In the present study, we compared the effect of the selective iNOS inhibitor aminoguanidine and the nonselective NOS inhibitor $\text{N}^\text{G}$-nitro-L-arginine methyl ester (L-NAME) on a dextran sulfate sodium (DSS)-induced model of colitis in rats. Experimental colitis was induced by a 3% DSS-solution added to drinking water for 7 days. Aminoguanidine (5~20 mg/kg) and L-NAME (10 mg/kg) were administered p.o. twice daily for the first 3 days, the last 3 days or all 6 days of DSS treatment. Body weight and severity of colitis (diarrhea, bloody feces) were observed over a period of 7 days. DSS treatment resulted in severe colonic lesions, accompanied by diarrhea, bloody feces, decrease of body weight and colon shortening. All of the parameters investigated improved significantly with aminoguanidine treatment at 20 mg/kg for 6 days or the last 3 days of DSS-treatment, but L-NAME did not significantly affect the colitis during these periods. When L-NAME or aminoguanidine was given in the first 3 days of DSS treatment, the colonic lesions were slightly aggravated by L-NAME but not affected by aminoguanidine. The expression of iNOS mRNA was observed from the 3rd day of DSS treatment. These results suggested that endogenous NO exerts a biphasic influence on DSS-induced colitis, depending on the NOS isoenzyme; a beneficial effect of NO derived from constitutive NOS and a detrimental effect of NO produced by iNOS in the development of colitis.

Key words: experimental colitis, nitric oxide, aminoguanidine, L-NAME, rat
INTRODUCTION

Although the etiology of ulcerative colitis remains unknown, a dysregulated immune response to normal bacterial microflora is likely an important factor in the pathogenesis of the disease (1). The uncontrolled activation of the immune system results in an overproduction of reactive oxygen and nitrogen metabolites (2). A high concentration of nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is associated with ulcerative colitis (3), and the level of iNOS-derived NO correlates with disease activity in ulcerative colitis (4). Moreover, the expression of iNOS is reportedly up-regulated in Crohn's disease as well as in experimental colitis models (5). However, the role NO plays in the pathophysiology of inflammatory bowel diseases remains controversial, and it is the subject of debate (3,5). While numerous animal studies have described an improvement of experimental colitis with iNOS inhibition (6,7), there are also reports showing the ineffectiveness (8,9) or the detrimental effect of those inhibitors (10-11). Likewise, there have been conflicting reports about whether NO prevents or aggravates injury in colitis or is only an inflammatory marker (12).

Ulcerative colitis induced by dextran sulfate sodium (DSS) is a widely used experimental model of inflammatory bowel diseases. The aim of the present study was to compare the effect of the relatively selective iNOS inhibitor aminoguanidine and the constitutive NOS (cNOS) inhibitor N\textsuperscript{6}-nitro-L-arginine methyl ester (L-NAME) on the DSS-induced model of colitis in rats, in an attempt to investigate the role of NO in the development of colonic lesions.

MATERIALS AND METHODS

Animals

Male Wistar rats (150~170 g, Shizuoka, Japan) were used. The animals were fed with standard rat chow and tap water ad libitum. All experimental procedures described were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University. All studies were carried out using 5~6 animals per group under unanesthetized conditions, unless otherwise specified.

Induction of Ulcerative Colitis

Dextran sulfate sodium (DSS), with a molecular weight of 5000 and sulfur content of 15-20%, was administered by adding it to water bottles in a 3% solution (w/v). Free access to DSS-containing water was given to animals for 7 days. Normal animals received tap water without containing DSS. The experiments were performed in the following groups: normal animals receiving water, vehicle-treated controls, aminoguanidine-treated animals (5, 10 and 20 mg/kg for 6 days during DSS-treatment, 20 mg/kg in the first 3 or last 3 days of DSS treatment, and L-NAME-treated rats (10 mg/kg given for 6 days during DSS-treatment, 10 mg/kg in the first 3 or last 3 days of DSS treatment). Aminoguanidine or L-NAME was given orally twice daily for 3 or 6 days during DSS treatment. Control animals received the vehicle (saline) in place of the active agent.
Evaluation of Colonic Damage

The animals were killed 7 days after the onset of DSS-treatment. To delineate the mucosal damage, 1 ml of 1% Evans blue was injected intravenously 30 min before sacrifice. The colon was excised and fixed with a 2% formalin solution for 10 minutes. After its immersion in formalin the large bowel was opened along the mesenteric attachment and examined macroscopically under a dissecting microscope (x10), for edema, hyperemia, erosion and ulceration. The area of macroscopically visible lesions was measured using NIH image 1.61 (NIH, Bethesda, MD, USA). The length of the colon (mm) was also determined. In addition, a disease activity index was determined macroscopically, according to the following criteria first reported by Cooper et al. (13): diarrhea score (0: normal stool; 1: mildly soft stool; 2: very soft stool; 3: watery stool) and bloody feces score (0: normal color stool; 1: brown color stool; 2: reddish color stool; 3: bloody stool).

Histological Evaluation

The distal colon was examined with a light microscope. The tissue samples were immersed in 10% formalin, sectioned at 5 µm, and stained with hematoxylin and eosin. The severity of tissue sections was graded microscopically as showing a normal appearance, mild infiltrates of small round cells, and polymorphonuclear leucocytes in the lamina propria mucosae with shallow or deep erosion/ulceration.

Determination of iNOS and eNOS mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from rat colonic mucosa was extracted using Sepasol RNA I (Nacalai Tesque, Kyoto, Japan). Total RNA primed by oligo (dT20) was reverse-transcribed with a first strand cDNA synthesis kit (Rever-Tra Ace alpha Toyobo, Japan) after treatment with DNasel (Toyobo, Japan) to remove contamination from genomic DNA. The PCR primers for rat iNOS were sense (5'-AACCCTGCTTGCTGACTTGCTGAA-3') and antisense (5'-GATGTTTGTAGCGCTGTGTGTC A-3') (651 bp) while those for rat eNOS were sense (5'-GCCTGTGAAACTTTCTGTGG-3') and antisense (5'-TATGCCAGCAGCACAGTCACTAGTA-3') (770 bp). An aliquot of the RT reaction product served as a template in 30 cycles with 0.5 min of denaturation at 95°C and 1 min of extension at 68°C using the Advantage 2 polymerase mixture (CLONTECH) on a thermal cycler (TAKERA TP-240). A portion of the PCR mixture was electrophoresed in 1.8 % agarose gel in TEA buffer (40 mM Tris 2 mM EDTA, and 20 mM acetic acid; pH: 8.1), and the gel was stained with ethidium bromide and photographed. The PCR product was subcloned into pGEM-T easy vector (Promega), and the sequence was determined by the dideoxy chain termination method with a Big Dye terminator v3.0 Cycle Sequencing kit (Biosystems), using an automatic DNA sequencer (ABI Prism 310 Genetic Analyser; Biosystems). The sequence of the PCR product was analyzed using the BLAST program (NBCI).

Preparation of Drugs

The drugs used were dextran sulfate sodium (DSS: Wako, Osaka, Japan), aminoguanidine, Nω-nitro-L-arginine methyl ester (L-NAME)(Sigma, St. Louis, USA) and Evans blue (Merck, Darmstadt, Germany). Aminoguanidine and L-NAME were dissolved in 0.9% saline solution. Evans blue was dissolved in distilled water. All drugs were prepared immediately before use and administered orally in a volume of 0.5 ml/100 g body weight or intravenously in a volume of 0.1 ml/100 g body weight.
Statistics

Data are presented as the mean±SE of 5 to 6 rats per group. Statistical analysis was performed using the two-tailed Dunnett's multiple comparison test, and values of p<0.05 were considered significant.

RESULTS

Effect of Aminoguanidine and L-NAME on DSS-Induced Ulcerative Colitis

Mucosal lesions: The animals treated with 3% DSS for 7 days developed symptoms of acute colitis at an incidence of 100%. Diarrhea was first detected on the 3rd day, bloody feces on the 4th day of treatment. Colitis was accompanied by severe loss of body weight, and the lesion score was 320.5±26.8 mm² in this group. Treatment with aminoguanidine for 6 days ameliorated the DSS-induced ulcerative colitis in a dose-dependent manner, with suppression of diarrhea and rectal bleeding as well as the loss of body weight (Tab. 1 and Fig. 1). The mucosal damage score in the groups treated with 5, 10 and 20 mg/kg of aminoguanidine was 278.8±29.3, 255.1±88.1, 171.3±35.7 mm², respectively, the effect at 20 mg/kg being significantly weaker as compared to the control. Likewise, this agent given at 20 mg/kg in the last 3 days of DSS treatment, also showed a significant reduction in the severity of lesions compared to the control, the damage score being 186.4±28 mm² (Fig. 2). This treatment in the final 3 days also significantly prevented the loss

Table 1. Body Weight Gain and Incidence of Diarrhea and Rectal Bleeding in Rats after 7 Days DSS-Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Body weight gain(%)</th>
<th>Incidence of Diarrhea</th>
<th>Incidence of Rectal bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5</td>
<td>10.52±4.36</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>-14.95±4.89</td>
<td>6/6</td>
<td>5/6</td>
</tr>
<tr>
<td>AG 5 mg/kg for 6 days</td>
<td>6</td>
<td>-9.56±3.34</td>
<td>6/6</td>
<td>4/6</td>
</tr>
<tr>
<td>AG 10 mg/kg for 6 days</td>
<td>6</td>
<td>-7.53±4.26</td>
<td>5/6</td>
<td>3/6</td>
</tr>
<tr>
<td>AG 20 mg/kg for 6 days</td>
<td>6</td>
<td>-3.88±5.61</td>
<td>3/6</td>
<td>2/6</td>
</tr>
<tr>
<td>AG 20 mg/kg for first 3 days</td>
<td>6</td>
<td>-10.55±3.5</td>
<td>6/6</td>
<td>5/6</td>
</tr>
<tr>
<td>AG 20 mg/kg for last 3 days</td>
<td>6</td>
<td>-4.1±2.53</td>
<td>3/6</td>
<td>3/6</td>
</tr>
<tr>
<td>L-NAME 10 mg/kg for 6 days</td>
<td>6</td>
<td>-10.4±4.5</td>
<td>5/6</td>
<td>5/6</td>
</tr>
<tr>
<td>L-NAME 10 mg/kg for first 3 days</td>
<td>6</td>
<td>-14.3±3.25</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>L-NAME 10 mg/kg for last 3 days</td>
<td>6</td>
<td>-11.53±5.22</td>
<td>6/6</td>
<td>5/6</td>
</tr>
</tbody>
</table>

Experimental colitis was induced in rats with a 3% DSS-solution added to drinking water for 7 days. Aminoguanidine (AG: 5-20 mg) or L-NAME (10 mg/kg) was administered p.o. twice daily for 6 days, the first 3 days, or the last 3 days of DSS-treatment. Data on body weight changes are presented as the mean±SE of 5~6 rats. Incidence of diarrhea or rectal bleeding means the number of animals showing >1 of the diarrhea score or the bloody feces score. Significant difference at p<0.05; a) from normal; b) from control.
of body weight gain with suppression of diarrhea and rectal bleeding (Tab. 1). On the other hand, L-NAME given at 10 mg/kg for 6 days or for the last 3 days during DSS treatment did not affect the experimental colitis. Moreover, this agent given in the first 3 days of DSS treatment caused a slight, though not significant, aggravation of colitis, the damage score being 358.5±26 mm$^2$ (Tab. 1 and Fig. 3).

Colon length: After 7 days treatment with DSS in drinking water, the colon was shortened in all animals. The colon length of DSS-treated control animals was 91.4±3.4 mm, significantly less than that of normal rats (127.5±8.0 mm) (Fig. 4 and 5). The DSS-induced shortening of the colon was improved in a dose-dependent manner following administration of aminoguanidine (5~20 mg/kg) for 6 days, and a significant effect was observed at the highest dose, the value being 105.4±4.4 mm. Similarly, aminoguanidine given at 20 mg/kg in the last 3 days of DSS treatment also restored the colon's length to 106.0±2.3 mm, which is significantly greater than that in the control group. By contrast, L-NAME given at 10 mg/kg for either all 6 days, the first 3 days or the last 3 days of DSS treatment, did not influence the colon shortening (Fig. 5 and 6).

Histology: By histological examination, no pathological change was detected in the colonic mucosa of normal animals. However, the DSS-treated control rat mucosa showed edema, deep erosions, ulcerations and infiltrates of polymorphonuclear leucocytes as well as lymphocytes. The microscopical grading in the severity of tissue damage was remarkably reduced by aminoguanidine but not by L-NAME, when they were given for 6 days during DSS treatment. In the
Figure 2. Effect of aminoguanidine (AG) on colonic ulceration area in DSS-induced rat ulcerative colitis. Experimental colitis was induced in rats with a 3% DSS-solution added to drinking water for 7 days. AG (5–20 mg/kg) was administered p.o. twice daily for all 6 days or the first 3 days or last 3 days of DSS-treatment. Data are presented as the mean±SE for 5–6 rats. * Significant difference from control, at p<0.05.

Figure 3. Effect of L-NAME on colonic ulceration area in DSS-induced rat ulcerative colitis. Experimental colitis was induced in rats with a 3% DSS-solution added to drinking water for 7 days. L-NAME (10 mg/kg) was administered p.o. twice daily for all 6 days or the first 3 days or last 3 days of DSS-treatment. Data are presented as the mean±SE for 5–6 rats.
Figure 4. Effect of aminoguanidine (AG) on colon length in DSS-induced rat ulcerative colitis. Experimental colitis was induced in rats by 3 with a 3% DSS-solution added to drinking water for 7 days. AG (5–20 mg/kg) was administered p.o. twice daily for all 6 days or the first 3 days or last 3 days of DSS-treatment. Data are presented as the mean±SE for 5–6 rats. Significant difference at p<0.05; # from normal; * from control.

Figure 5. Effect of L-NAME on colon length in DSS-induced ulcerative colitis. Experimental colitis was induced in rats with a 3% DSS-solution added to drinking water for 7 days. L-NAME (10 mg/kg) was administered p.o. twice daily for all 6 days or the first 3 days or last 3 days during DSS-treatment. Data are presented as the mean±SE for 5–6 rats. # Significant difference from normal rats, at p<0.05.
Figure 6. Gross appearances of colonic lesions induced by DSS in rats. Colitis was induced in rats with a 3% DSS-solution added to drinking water for 7 days. Aminoguanidine (AG: 20 mg/kg) or L-NAME (10 mg/kg) was administered p.o. twice daily for 6 days. Note that DSS treatment caused a marked decrease in the colon length, and that this shortening was improved by treatment with AG but not L-NAME.

<table>
<thead>
<tr>
<th>Normal</th>
<th>3% DSS</th>
<th>3% DSS + AG (20 mg/kg)</th>
<th>3% DSS+L-NAME (10 mg/kg)</th>
</tr>
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<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Control (3% DSS)</td>
<td>DSS+AG (20 mg/kg)</td>
<td>DSS+L-NAME (10 mg/kg)</td>
</tr>
</tbody>
</table>

Figure 7. Histology of rat colonic mucosa; A: normal rat, B: 3% DSS-treated control, C: 3% DSS+20 mg/kg of aminoguanidine (AG), D: 3% DSS+10 mg/kg of L-NAME. Experimental colitis was induced in rats with a 3% DSS-solution added to drinking water for 7 days. AG (20 mg/kg) or L-NAME (10 mg/kg) was administered p.o. twice daily for 6 days.
rats treated with 20 mg/kg of aminoguanidine, the colonic mucosa showed only slight pathologic changes, which markedly contrasted to the control rat mucosa (Fig. 7). The histological findings paralleled the macroscopically visible damage.

**Expression of iNOS and eNOS mRNA in Rat Colon**

While the expression of eNOS mRNA was observed in the normal rat colon, the gene expression of iNOS was not detected in the mucosa. However, the expression of iNOS mRNA was found to occur in the colonic mucosa from the 4th day of DSS treatment and became much greater on day 6 during the treatment (Fig. 8). The eNOS mRNA proved to be expressed steadily in DSS-treated rats.

**DISCUSSION**

It is known that DSS-induced experimental colitis has similar histological and pathological characteristics to human ulcerative colitis (14). The present study

*Figure 8. RT-PCR analysis for iNOS and eNOS mRNA in rat colonic mucosa during DSS treatment. The expression of iNOS and eNOS mRNA was determined in normal rat 2, 4, and 6 days after the onset of DSS treatment.*
demonstrated that the selective iNOS inhibitor aminoguanidine afforded a protective effect on a DSS-induced colitis in rats when given for all 6 days or for the last 3 days of DSS treatment, but was ineffective when given only for the first 3 days. We also found that the nonselective NOS inhibitor L-NAME administered for all 6 days or last 3 days of DSS treatment did not affect colonic inflammation in this rat model. Furthermore, L-NAME given in the first 3 days of DSS treatment tended to aggravate the colonic inflammation and lesions. These results clearly indicate a dual role for endogenous NO in the development of ulcerative colitis induced in rats by DSS; a protective effect by cNOS/NO and a detrimental effect by iNOS/NO.

Ulcerative colitis is an inflammatory disease confined to the mucosa and, to a lesser extent, the adjacent submucosa. Although the cause and developmental process of this disease remain unknown, the characteristics of the disease are shared by other inflammatory disorders of the gut, ie., activated neutrophils and macrophages are the major components of the active lesions in ulcerative colitis (15,16). Large numbers of neutrophils and macrophages enter the injured mucosa during acute inflammation, leading to an overproduction of oxygen radicals and NO (17). Both iNOS expression and NO production are reportedly increased in the epithelial cells and the tissue macrophages of the inflamed mucosa in patients with inflammatory bowel diseases (18,19). The interaction of oxygen radicals and NO results in the formation of cytotoxic nitrogen species, peroxynitrite, which is known to induce protein nitration as well as lipid peroxidation (20). Indeed, the severity of DSS-induced colitis was significantly attenuated in mice genetically deficient in iNOS (21). Similar results were obtained by Kriegstein et al. (22), who reported that murine intestinal inflammation was prevented by treatment with a highly selective iNOS inhibitor (1400 W).

However, NO has an important role also in the normal physiology of the colon (19). In fact, the effects of NO and NOS inhibitors on colitis are controversial (3,5). Some authors consider NO as an inflammatory marker, and reported the importance of this free radical in the pathogenesis of experimental colitis (12). We observed in the present study that the selective iNOS inhibitor aminoguanidine significantly reduced the severity of colonic lesions when administered for all 6 days or for the last 3 days of DSS-treatment. This agent also prevented the colon from shortening and ameliorated the histological inflammation in this colitis model. By contrast, the nonselective NOS inhibitor L-NAME tended to aggravate the colonic lesions when given for the first 3 days of DSS treatment, although it had no effect when given for all 6 days or the last 3 days. These results together with the outcome of aminoguanidine treatment suggest a paradoxical role for NO derived from iNOS and cNOS in the development of experimental colitis. Endogenous NO has a dual influence on DSS-induced colitis, depending on the NOS isozyme; the NO produced by iNOS in the final 3 days of DSS treatment is likely detrimental, while that derived from cNOS is considered to be beneficial in this model of colitis. Indeed, we observed
the expression of iNOS mRNA in the colon from 3 days after the onset of DSS treatment but not in the normal rat colon, although eNOS, an isozyme of cNOS, was observed in both normal and DSS-treated rat colon. Sasaki et al. (23) reported an increase in the disease activity of colitis induced in eNOS-deficient mice by DSS treatment.

A dual role for NO has been reported in the pathogenesis of other lesion models in the gastrointestinal tract (24-28). Several studies showed a protective influence of NO against aspirin- or stress-induced gastric lesions (24,25), while others reported a detrimental effect of this substance on the development of gastric lesions induced by compound 48/80 (26). Especially, Tanaka et al. (28) demonstrated a biphasic role of endogenous NO in the pathogenesis of indomethacin-induced small intestinal lesions. These lesions were prevented by pretreatment with aminoguanidine or later administration of L-NAME, while L-NAME given prior to indomethacin actually increased the severity of lesions. A similar finding was observed using a rat colitis model by Kiss et al. (29), who showed that although pretreatment with L-NAME augmented the colonic injury induced by an intracolonic challenge with trinitrobenzene sulfonic acid (TNBS), the later administration of L-NAME 6 hr after the TNBS challenge, when iNOS is detected, reduced the tissue injury. Likewise, the microvascular injury induced in the rat jejunum by indomethacin was prevented by later administration of L-NAME, while the concurrent administration of L-NAME augmented the intestinal plasma leakage caused by indomethacin (30). On the basis of these findings, it is assumed that nonselective NOS inhibitors, depending upon the time of administration, exhibit a beneficial or detrimental effect on gut inflammation, including small intestinal damage induced by indomethacin and colonic damage induced by TNBS or DSS. It should be noted in the present study that L-NAME given for the last 3 days of DSS treatment did not have a clear beneficial influence on the colonic lesions. Because L-NAME inhibits iNOS activity, similar to aminoguanidine, this agent should have ameliorated the severity of colonic lesions. However, L-NAME did not improve the outcome of colonic lesions as was observed by the treatment with aminoguanidine. Thus, this result does not support a pathogenic role for iNOS/NO in this colitis model. We do not have any explanation for this result at present, yet it may be assumed that an optimal level of NO is necessary for maintaining the integrity of the colonic mucosa, with levels that are too high as well as too low likely to be harmful in this tissue. Since L-NAME inhibits both cNOS and iNOS activity, it might be possible that the amelioration effect brought about by iNOS inhibition is masked by the aggravating effect due to cNOS inhibition.

Given the findings of the present study, we concluded that aminoguanidine, the selective iNOS inhibitor, affords a prophylactic effect on DSS-induced colitis in rats. This study also indicated that NO produced endogenously plays a dual role in the modulation of the inflammatory process, depending on the NOS isoform; with cNOS/NO having a beneficial influence and iNOS/NO having a
detrimental effect. Further study is required to elucidate the mechanisms by which NO produced by iNOS aggravates colonic lesions.

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