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The inducible nitric oxide synthase inhibitor ONO-1714 blunts dextran sulfate sodium colitis in mice

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Abstract

In mice with acute dextran sulfate sodium colitis, we examined the effect of inducible nitric oxide synthase inhibition by (1*S*,5*S*,6*R*,7*R*)-7chloro-3-amino-5methyl-2-azabicyclo[4.1.0]heptane hydrochloride (ONO-1714) on colonic biochemistry, injury, and inflammation. Colonic luminal nitrate and nitrite were measured by the Griess reaction; inducible nitric oxide synthase messenger RNA expression by reverse transcription-polymerase chain reaction; and nitrotyrosine by immunohistochemistry. Mice with colitis showed increases in nitrate and nitrite, inducible nitric oxide synthase messenger RNA, and numbers of cells staining for nitrotyrosine. Colonic inflammation was severe. ONO-1714 inhibited increases in nitrate and nitrite and numbers of nitrotyrosine-positive cells; injury and inflammation also were reduced. Dextran sulfate sodium-induced increases in thiobarbituric acid-reactive substances, a lipid peroxidation marker, were blunted by ONO-1714, which also inhibited increases in mucosal inflammatory cytokines. Nitric oxide produced by inducible nitric oxide synthase may contribute to colonic inflammation by nitrosation, oxidative damage, and enhanced inflammatory cytokines.

Keywords: Dextran sulfate sodium; Nitric oxide (NO); Nitric oxide (NO) synthase, inducible; ONO-1714; (Mouse)

1. Introduction

Nitric oxide has been implicated in the pathogenesis of inflammatory bowel disease including ulcerative colitis and Crohn's disease. Nitric oxide production as well as expression of inducible nitric oxide synthase in intestinal mucosa are enhanced in active human inflammatory bowel disease (Singer et al., 1996; Rachmilewitz et al., 1995, 1998; Kimura et al., 1998; Dijkstra et al., 1998), experimental intestinal inflammation induced by exogenous agents (Kankuri et al., 1999; Miampamba and Sharkey, 1999), and spontaneous colitis in genetically altered rodents (Aiko and Grisham, 1995; Harren et al., 1998). According to immunohistochemistry, inducible nitric oxide synthase is expressed intensely in inflamed colonic epithelium in ulcerative colitis and Crohn's disease, as well as polymorphonuclear leukocytes within the colonic lumen and in crypt abscesses, and some mononuclear cells in the inflamed lamina propria (Singer et al., 1996; Kimura et al., 1998). Thus, nitric oxide synthesis may be augmented in inflammatory bowel disease and influence the disease process. One proposed mechanism for nitric oxide-induced cytotoxicity is interaction with superoxide to produce peroxynitrite, an oxidizing agent that initiates lipid peroxidation, sulfhydryl oxidation of proteins, and nitration of aromatic amino acids. Nitrotyrosine immnunoreactivity, an index of nitrosative stress, has been observed not only in the lamina propria but also in the surface epithelium in experimental colitis (Zingarelli et al., 1998, 1999) and in patients with inflammatory bowel disease (Singer et al., 1996; Kimura et al., 1998).

A growing body of evidence indicate that in small amounts produced under normal or acute inflammatory conditions by constitutive nitric oxide synthase, nitric oxide may act as an important endogenous inhibitor of inflammation; however, larger quantities of nitric oxide associated with inducible nitric oxide synthase activity in chronic inflammatory conditions such as inflammatory bowel disease may be detrimental to intestinal integrity.

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Accordingly, the relative lack of specificity of nitric oxide synthase inhibitors has produced conflicting results in inhibition experiments; in some studies inhibition attenuated experimental colitis (Miller et al., 1993; Grisham et al., 1994; Southey et al., 1997; Aiko et al., 1998; Nakamura et al., 1999), while in others inhibition showed little effect (Hogaboam et al., 1995), or even exacerbation (Pfeiffer and Qiu, 1995).

We used a dextran sulfate sodium colitis model in mice that is histologically similar to human ulcerative colitis, showing focal crypt lesions, mucosal and submucosal inflammation, and granulocytic infiltration (Okayasu et al., 1990). Recently, Obermeier et al. (1999) demonstrated that aminoguanidine, a relatively selective inhibitor of inducible nitric oxide synthase, aggravated an acute form of dextran sulfate sodium colitis but improved a chronic form. ONO-1714, a novel cyclic amidine analogue, inhibits human inducible nitric oxide synthase with an inhibition constant of 1.88 nM and inhibits the rodent enzyme with similar potency (Naka et al., 2000). The inhibitory activity of ONO-1714 (50% inhibitory concentration; 4.0 nM) is 875 times of that N^G-monomethyl-L-arginine (L-NMMA) and 4900 times that of aminoguanidine (Naka et al., 2000). In terms of selectivity, ONO-1714 is approximately 34 and 2 times more selective for inducible nitric oxide synthase than L-NMMA and aminoguanidine, respectively. Therefore, ONO-1714 is a promising tool for clarifying the role of inducible nitric oxide synthase in disease and may have considerable therapeutic potential. In the present study, we used ONO-1714 to determine the involvement of inducible nitric oxide synthase in dextran sulfate sodium colitis in mice.

2. Materials and methods

2.1. Chemicals

All chemicals were prepared immediately before use. The selective inhibitor (1*S*,5*S*,6*R*,7*R*)-7chloro-3-amino-5-methyl-2-azabicyclo[4.1.0]heptane hydrochloride (ONO-1714) was a gift from Ono Pharmaceutical (Osaka, Japan). Thiobarbituric acid and 3,3′,5,5′-tetramethylbenzidine were obtained from Wako (Osaka, Japan). We obtained 1,1,3,3-tetramethoxy propane from Tokyo Kasei (Tokyo, Japan). Enzyme-linked immunosorbent assay for interleukin-4 and -10 as well as interferon-γ were obtained from BioSource International (Camarillo, CA). All other chemicals used were of reagent grade.

2.2. Animals

Nine-week-old female BALB/c mice weighing 18 to 20 g were obtained from Keari (Osaka, Japan). Mice were housed in our animal quarters prior to experiments, and were maintained at 18°C to 24°C with a 12-h light/dark cycle. They were fed a standard diet (Oriental Yeast, Tokyo, Japan) and water ad libitum. Mice were anesthetized with urethane (1 mg/kg, i.p.) before they were killed by exsanguination from the abdominal aorta and colons were removed.

2.3. Induction of colitis and design of treatment

Acute colitis was induced by giving 8% dextran sulfate sodium (molecular weights; 8000; lot no. DS-605, Seikagaku, Tokyo, Japan) orally in drinking water for 7 days. For kinetic study, mice were killed on day 0, 3, 5, or 7. ONO-1714 was dissolved in physiologic saline. Mice were randomized into groups receiving different concentrations of ONO-1714 (or only the physiologic saline vehicle) by intraperitoneal injection for 7 days. Mice were killed on day 7, and colons were removed for the histologic examination, biochemical assay, and RNA isolation. Colonic bleeding was quantified indirectly as hemoglobin concentration in luminal lavage fluid using the kit manufacturer's protocol (Wako).

2.4. Nitrate / nitrite assay

Nitric oxide production was quantified indirectly as nitrite plus nitrate concentration in luminal lavage fluid and plasma. Luminal lavage fluid was centrifuged, and the supernatant was aliquoted and frozen until assay. Plasma was centrifuged for 45 min at $2000 \times g$ with an ultrafilter (Centricon, Millipore, Bedford, MA). Nitrate in the supernatant was reduced to nitrite by incubation with nitrate reductase and NADPH at room temperature for 1 h. Nitrite concentration in the reduced samples was measured by the Griess reaction according to the kit manufacturer's protocol (Dojin, Kumamoto, Japan). Nitrite concentrations were calculated by comparison with standard solutions of sodium nitrate prepared in saline solution after reduction.

2.5. Immunohistochemistry for nitrotyrosine

Tyrosine nitration was evaluated in colonic sections by immunohistochemistry according to the kit manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY). Paraffin-embedded colonic specimens were sectioned, deparaffinized, and rehydrated. Sections then were placed in a Coplin jar for antigen retrieval in 10 mM citrate acid (pH 6). The Coplin jar was placed in a dish filled with water and treated in a 700-W microwave oven at the high setting for 2 to 3 min. Rinsed slides were treated with 2% hydrogen peroxide for 5 min to block endogenous peroxidase activity, and then were rinsed briefly in phosphate-buffered saline. Nonspecific binding was blocked by incu-

bating the slides with phosphate-buffered saline/1% bovine serum albumin for 1 h at room temperature. Sections then were incubated with 5 μg/ml antinitrotyrosine antibody or with control solutions at room temperature for 1 h. Diaminobenzidine was used as a chromogen and light green acidified with 0.01% acetic acid was used as a counterstain.

2.6. RNA analysis

Samples for messenger RNA isolation were removed from the colon. Total RNA was isolated with the acid guanidinium phenol chloroform method using an ISOGEN kit (Nippon Gene, Tokyo, Japan). The concentration of RNA was determined by absorbance at 260 nm in relation to absorbance at 280 nm. The RNA was stored at −70°C until reverse transcription was performed. One microliter of reverse transcription product was added to 3 mM concentrations of primers for inducible nitric oxide synthase and β-actin (as an internal standard). In a solution containing 0.5 U of *Taq* DNA polymerase (Takara Biochemicals, Shiga, Japan) in a final volume of 50 μl, primers were as follows: for inducible nitric oxide synthase, sense 5′-TCACTGGGACAGCACAGAAT-3′, and antisense 5′-TGTGTCTGCAGATGTGCTGA-3′; for β-actin, sense 5′-TGTGATGGTGGGAATGGGTCAG-3′, and antisense 5′-TTTGATGTCACGCACGATTTCC-3′. The mixture was subjected to polymerase chain reaction amplification for 30 cycles (1 min at 94°C, 1 min at 54°C, and 1 min at 72°C). Reaction products were separated electrophoretically on a 2.5% agarose gel and stained with ethidium bromide.

2.7. Evaluation of colonic inflammation

Colonic inflammation was evaluated in terms of by body weight, total length of the colon, luminal hemoglobin, and histology. For histologic evaluation, formalin fixed tissues were stained with hematoxylin and eosin and evaluated microscopically by a pathologist unaware of the experimental conditions for any given specimen.

2.8. Measurements of myeloperoxidase activity and thiobarbituric acid-reactive substances

As an index of lipid peroxidation, the concentration of thiobarbituric acid-reactive substances was measured in colonic mucosa using the method of Ohkawa et al. (1986). Colonic mucosa was scraped from the deeper layers using two glass slides and homogenized with 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl in a Teflon Potter-Elvehjem homogenizer. Thiobarbituric acid-reactive substances in mucosal homogenates were expressed as nanomoles of malondialdehyde per milligram of protein using 1,1,3,3-tetramethoxypropane as a standard. Total protein in tissue homogenates was measured by the method of Lowry et al. (1951).

As an index of neutrophil accumulation, tissue-associated myeloperoxidase activity was determined by a modification of the method of Grisham et al. (1986). Two milliliters of mucosal homogenate were centrifuged at $20,000 \times g$ for 15 min at 4°C to pellet insoluble cellular debris. The pellet then was rehomogenized in an equivalent volume of 0.05 M potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide. Samples were centrifuged at $20,000 \times g$ for 15 min at 4°C, and supernatants were saved. Myeloperoxidase activity was assessed by measuring H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of myeloperoxidase that caused a change in absorbance of 1.0/min at 655 nm and 25°C.

2.9. Determination of colonic content of inflammatory cytokines

Concentrations of inflammatory cytokines (interleukin-4, interleukin-10, and interferon-γ) in the supernatant of mucosal homogenates were determined by enzyme-linked immunosorbent assay kits with antibodies specific for the respective mouse cytokines.

2.10. Statistics

The results are presented as the mean ± S.E.M. Data were compared by one-way analysis of variance. Differences were considered significant if the P value was less than 0.05 based on Dunnett's multiple comparison test. All analyses were performed using Stat View 5.0-J program (Abacus Concepts, Berkeley, CA) with a Macintosh computer.

Table 1
Effects of ONO-1714 on body weight, colon length, and intraluminal hemoglobin in dextran sodium sulfate-induced colitis in mice

Group	Weight gain (g/7 days)	Colon length (cm)	Intraluminal hemoglobin (mg/cm)
No colitis	1.18 ± 0.44	10.98 ± 0.24	0.86 ± 0.07
Dextran sulfate sodium only	−2.00 ± 0.06[a]	8.98 ± 0.09[a]	4.22 ± 1.27[b]
ONO-1714 coadministration			
0.03 mg/kg	−0.73 ± 0.17[c]	9.80 ± 0.12[c]	0.90 ± 0.07[c]
0.1 mg/kg	−0.95 ± 0.10[c]	9.77 ± 0.15[c]	1.16 ± 0.14[c]
0.3 mg/kg	−0.63 ± 0.13[d]	9.98 ± 0.13[d]	1.33 ± 0.15[c]

Each value indicates the mean ± S.E.M. for five mice. ONO-1714 dissolved in saline was administered to mice by intraperitoneal injection.

[a] $P < 0.05$ and [b] $P < 0.01$ compared to mice without colitis; [c] $P < 0.05$ and [d] $P < 0.01$ compared to mice receiving only dextran sulfate sodium.

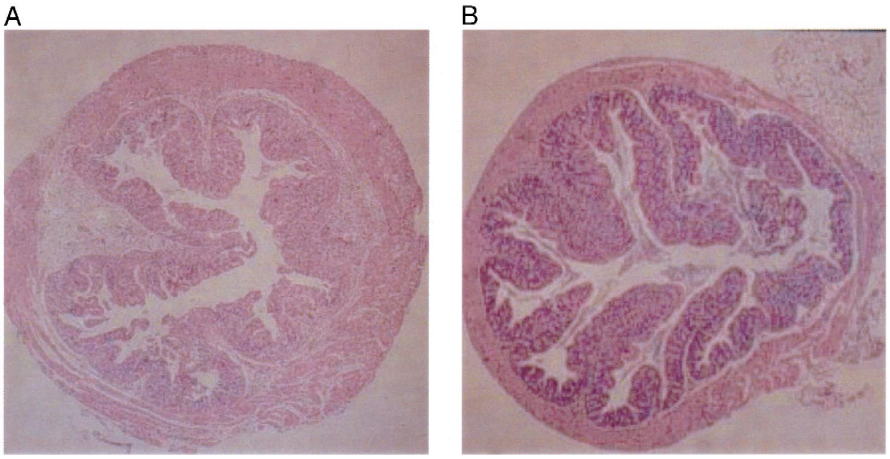

Fig. 1. Appearance of the colon in a dextran sulfate sodium-treated mouse (A) and a mouse also receiving ONO-1714-treated mice (B). Loss and shortening of crypts, mucosal erosions, inflammatory cell infiltration, and goblet cell depletion are seen in A. In B, smaller erosions are associated with less inflammatory cell infiltration. Hematoxylin and eosin, ×10.

2.11. Ethical consideration

Maintenance of animals and experimental procedures were carried out in accordance with the US National Institutes of Health guidelines for use of experimental animals. All experiments were approved by the Kyoto Prefectural University of Medicine Animal Care Committee (Kyoto, Japan).

3. Results

3.1. Effect of ONO-1714 on experimental colitis

Mice exposed to 8% dextran sulfate sodium developed symptoms of acute colitis. Diarrhea was observed first, followed by rectal bleeding and severe weight loss. The first dose–response study for the protective action of ONO-1714 (0.001–0.3 mg/kg) against dextran sulfate sodium-induced colitis showed that this compound inhibited the decrease of colon length with 50% inhibition at 0.04 mg/kg. Judging from these data, we set the doses of ONO-1714 in 0.03, 0.1, and 0.3 mg/kg and tested it. Decreases in body weight and shortening of the colon were significantly reversed by treatment with ONO-1714 at doses of 0.03, 0.1, and 0.3 mg/kg (Table 1). Lavage fluid hemoglobin concentrations were significantly elevated (approximately 5-fold) in the control group. This increase also was significantly avoided by ONO-1714 treatment.

The protective effect of ONO-1714 was confirmed histologically. Fig. 1 shows typical features in an ONO-1714

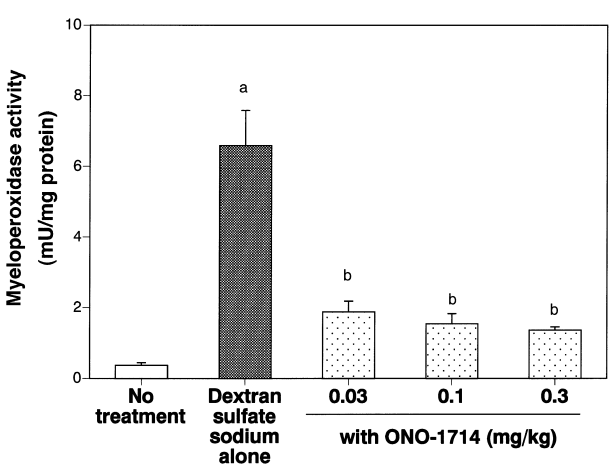

Fig. 2. Effect of ONO-1714 on neutrophil accumulation in the colonic mucosa of mice administered 8% dextran sulfate sodium. Values represent the mean + S.E.M. for six to eight mice. [a]$P < 0.05$ compared to untreated mice; [b]$P < 0.05$ compared to mice receiving only dextran sulfate sodium.

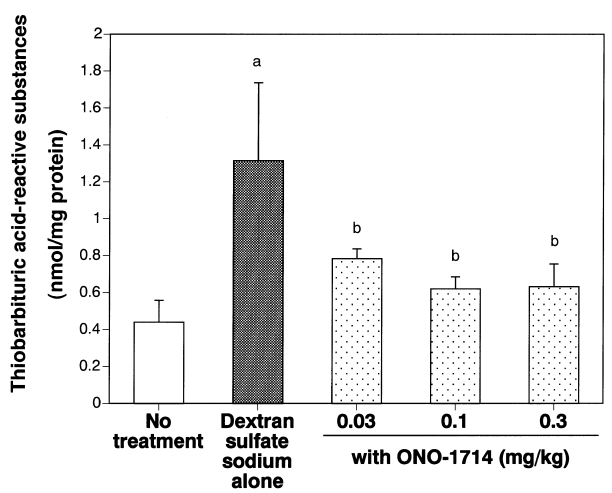

Fig. 3. Effect of ONO-1714 on the increase in lipid peroxide concentration in colonic mucosa of mice administered 8% dextran sulfate sodium. Values represent the mean + S.E.M. for six to eight mice. [a]$P < 0.01$ compared to untreated mice; [b]$P < 0.01$ compared to mice receiving only dextran sulfate sodium.

Table 2
Effects of ONO-1714 on nitrate and nitrite concentration of luminal lavage fluid and serum in dextran sulfate sodium-induced colitis in mice

Group	Nitrate plus nitrite	
	Luminal lavage fluid (nM/cm)	Serum (μM)
No colitis	0.46 ± 0.27	35.7 ± 9.8
Dextran sulfate sodium only	5.68 ± 1.89[b]	86.2 ± 2.9[a]
ONO-1714 coadministration		
0.03 mg/kg	1.24 ± 0.71[d]	61.8 ± 6.2
0.1 mg/kg	1.03 ± 0.48[d]	59.9 ± 7.2
0.3 mg/kg	0.65 ± 0.21[d]	54.9 ± 9.1[c]

Each value indicates the mean ± S.E.M. for five mice. ONO-1714 dissolved in saline was administered to mice by intraperitoneal injection.
[a] $P < 0.05$ and [b] $P < 0.01$ compared to mice without colitis; [c] $P < 0.01$ and [d] $P < 0.001$ compared to mice receiving only dextran sulfate sodium.

Table 3
Effects of ONO-1714 on mucosal content of inflammatory cytokines in dextran sodium sulfate-induced colitis in mice

Group	IL-4	IL-10 (pg/mg protein)	INF-γ
No colitis	6.67 ± 0.99	37.5 ± 6.1	27.9 ± 6.2
Dextran sulfate sodium only	21.93 ± 5.48[a]	97.6 ± 24.9[a]	70.0 ± 12.6[b]
ONO-1714 coadministration			
0.03 mg/kg	8.29 ± 1.05[c]	37.0 ± 3.9[c]	29.1 ± 0.9[c]
0.1 mg/kg	8.27 ± 0.46[c]	43.8 ± 6.6[c]	27.6 ± 5.7[c]
0.3 mg/kg	9.21 ± 1.34[c]	42.0 ± 4.8[d]	29.6 ± 2.5[c]

Each value indicates the mean ± S.E.M. for five mice. ONO-1714 dissolved in saline was administered to mice by intraperitoneal injection.
[a] $P < 0.05$ and [b] $P < 0.01$ compared to mice without colitis; [c] $P < 0.05$ and [d] $P < 0.01$ compared to mice receiving only dextran sulfate sodium.

(1.0 mg/kg) treated group and the control group. Administration of 8% dextran sulfate sodium alone for 7 days resulted in large areas of epithelial crypt loss, predominantly neutrophilic infiltrate throughout the mucosa, ulceration, and mucosal bleeding. In contrast, cotreatment with ONO-1714 resulted in smaller erosions with few neutrophils.

Neutrophil accumulation was also evaluated by the measurement of myeloperoxidase activity in colonic mucosal homogenates. This myeloperoxidase activity increased from a basal concentration of 0.37 ± 0.07 mU/mg protein to 6.59 ± 0.99 mU/mg protein at day 7 (Fig. 2). The increase in myeloperoxidase activity in the colonic mucosa with dextran sulfate sodium administration was significantly inhibited by cotreatment with ONO-1714 at doses of 0.03, 0.1, and 0.3 mg/kg.

3.2. Effect of ONO-174 on thiobarbituric acid-reactive substances

Thiobarbituric acid-reactive substances in colonic mucosa increased significantly from a basal concentration of 0.439 ± 0.118 to 1.314 ± 0.423 nmol/mg protein with 7 days of dextran sulfate sodium administration (Fig. 3). The increase in reactive substances in colonic mucosa was inhibited by ONO-1714 at doses of 0.03, 0.1, and 0.3 mg/kg (Fig. 3).

3.3. Effect of ONO-1714 on luminal and plasma nitrate and nitrite concentrations and nitrotyrosine formation

To test whether ONO-1714 inhibited nitric oxide production in vivo, we assessed luminal and serum concentrations of nitrate plus nitrite by the Griess reaction, and

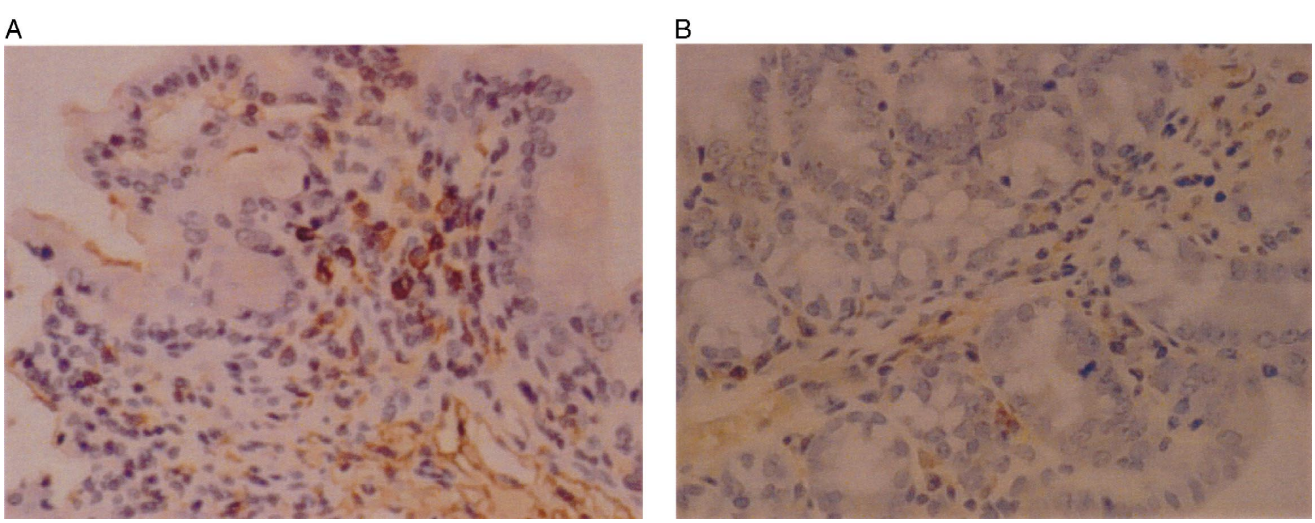

Fig. 4. Immunohistochemical demonstration of nitrotyrosine in inflamed colon from a mouse treated with only dextran sulfate sodium (A) and a mouse also receiving ONO-1714 (B). In A, extensive immunoreactivity is noted throughout the villus including epithelial cells, inflammatory cells, and interstitial tissues. In B, ONO-1714 cotreatment has markedly reduced immunohistochemical staining for nitrotyrosine. Magnification, ×100.

formation of nitrotyrosine by immunohistochemistry. Nitrate plus nitrite concentrations in the colonic lumen and in serum of mice with colitis were significantly elevated (approximately 12- and 2-fold, respectively) beyond concentrations in control mice receiving no dextran sulfate sodium (Table 2). These increases were significantly abolished by ONO-1714 cotreatment at doses of 0.03, 0.1, and 0.3 mg/kg (for the lumen) and of 0.3 mg/kg (for serum).

To evaluate the role of peroxynitrite formation in the present model, we performed immunohistochemistry for nitrotyrosine. Immunoreactivity for nitrotyrosine was evident throughout the gut wall in dextran sulfate sodium-induced colitis, being particularly strong in epithelial cells, neutrophils, and macrophages (Fig. 4). No staining was evident when the antibody was preincubated with the antigen, nitrotyrosine. Coadministration of ONO-1714 to mice greatly prevented this enhanced immunohistochemical staining for nitrotyrosine.

3.4. Effect of ONO-1714 on mucosal contents of inflammatory cytokines

The colonic content of inflammatory cytokines (interleukin-4, interleukin-10, and interferon-γ) was increased significantly 7 days after dextran sulfate sodium administration (Table 3). Increases in inflammatory cytokines in colonic mucosa were significantly inhibited by ONO-1714 coadministered at doses of 0.03, 0.1, and 0.3 mg/kg.

3.5. Effects of ONO-1714 on RNA expression for inducible nitric oxide synthase

To further analyze effects of ONO-1714 on experimental intestinal inflammation, we assessed colonic messenger RNA expression for inducible nitric oxide synthase using a semiquantitative reverse transcription-polymerase chain reaction yielding a 456 base pair product. As shown in Fig. 5, we found inducible nitric oxide synthase gene expression in control animals to be negligible or faint. In contrast, transcription was readily shown in dextran sulfate sodium-treated mice throughout the time course of the study (days 3 to 7 in all animals tested, with peak expression on day 7). Treatment with ONO-1714 suppressed messenger RNA expression of inducible nitric oxide synthase in inflamed colonic tissue (Fig. 5).

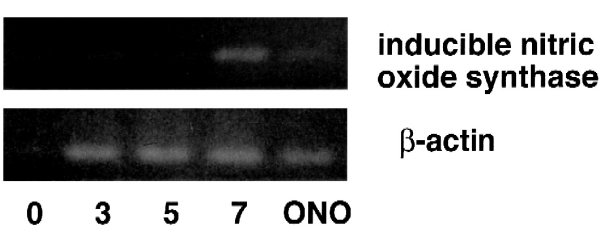

Fig. 5. Effects of ONO-1714 on RNA expression for inducible nitric oxide synthase in colonic tissues of mice given 8% dextran sulfate sodium. A representative 2% agarose gel of reverse transcription-polymerase chain reaction products is shown, also including β-actin messenger RNA for normal colon tissue (0), and inflamed tissue at 3 days (3), 5 days (5), and 7 days (7), and inflamed tissue cotreated with ONO-1714 at a dose of 1.0 mg/kg at 7 days (ONO).

4. Discussion

The present results clearly demonstrated nitric oxide overproduction accompanying increased inducible nitric oxide synthase messenger RNA expression during development of dextran sulfate sodium-induced colitis, while cotreatment with potent, selective inhibitor of this enzyme, ONO-1714, ameliorated the experimental colitis. Nitrate and nitrite in the gut lumen were used as an index of intestinal nitric oxide production. While other methods are more sensitive and questions exist as to source (nitric oxide vs. bacterial production), nitrate and nitrite in colitis were clearly elevated over those in control mice, in agreement with findings in human ulcerative colitis (Roediger et al., 1986) and in trinitrobenzenesulfonic acid colitis in rats (Miller et al., 1993). Inducible nitric oxide synthase is known to be regulated at the transcriptional level and can be induced by a variety of pro-inflammatory cytokines and mediators as well as by certain bacterial products. Not surprisingly, inducible nitric oxide synthase RNA expression was substantially upregulated in colitic tissues after 3, 5, and 7 days of dextran sulfate sodium administration. While our method of RNA amplification was not quantitative and the increases seen were relative, other studies corroborate our findings. For example, expression of inducible nitric oxide synthase has been observed in inflamed colonic tissue from patients with inflammatory bowel disease (Singer et al., 1996; McLaughlan et al., 1997), trinitrobenzenesulfonic acid-treated guinea pigs (Miller et al., 1995), and interleukin-2-deficient mice (Harren et al., 1998). In our study, increased expression of RNA encoding inducible nitric oxide synthase coincided with increased luminal nitric oxide production, which was significantly inhibited by ONO-1714 in a dose-dependent manner. In animals receiving ONO-1714 at a dose of 0.3 mg/kg, the luminal concentration of nitrates and nitrites decreased to the basal level seen in mice not receiving dextran sulfate sodium. Thus, we found protective effects against colitis via inhibition of inducible nitric oxide synthase in vivo.

Many types of nitric oxide synthase inhibitors have been described; some are L-arginine analogues. However, Southan and Szabo (1996) have stressed that L-arginine-based inhibitors may not be ideal when targeting a particular isoform (especially inducible nitric oxide synthase) because of limited selectivity and relatively low potency. While aminoguanidine is a relatively selective inhibitor of inducible nitric oxide synthase, its inhibitory activity is very weak (Hasan et al., 1993). Naka et al. (2000) recently reported that ONO-1714 is 875 times as more potent as

L-NMMA and over 4900 times as potent as aminoguanidine, and that ONO-1714 inhibited lipopolysaccharide-induced elevation of plasma nitrite and nitrate in mice with a 50% inhibitory dose of 0.01 mg/kg s.c. These data indicate that ONO-1714 can help to isolate the contribution of inducible nitric oxide synthase in in vivo disease models. In our present study, intestinal injury was assessed by a variety of methods including length of the colon, luminal content of hemoglobin, granulocytic infiltration, and histologic findings. In each assessment, animals receiving dextran sulfate sodium plus ONO-1714 could not be distinguished from control mice without colitis. These findings suggest that induction of inducible nitric oxide synthase is an early and critical event in this mouse model of inflammatory bowel disease. We have continued to investigate the role of this enzyme in other animal models of inflammatory bowel disease using ONO-1714.

One mechanism by which the nitric oxide overproduction may promote inflammatory injury is via an ability to mediate chemotaxis of both granulocytes and monocytes. A previous report suggested that nitric oxide may enhance interleukin-8 release in a cultured human endothelial cell line; thus, nitric oxide may enhance neutrophil chemotaxis in the intestine via an interleukin-8-dependent mechanism (Villarete and Remick, 1995). Inhibition of nitric oxide synthase activity has been shown to inhibit chemotaxis by neutrophils and monocytes in vitro. In the present study, an increase in colonic myeloperoxidase activity, indicating neutrophilic infiltration, was significantly inhibited by treatment with ONO-1714. This effect is consistent with the finding of Hogaboam et al. (1995), in which colonic myeloperoxidase was reduced in rats with trinitrobenzenesulfonic acid that were treated orally with N^{ω}-nitro-L-arginine methylester but is in disagreement with the results of Southey et al. (1997), where an inducible nitric oxide synthase inhibitor, *S*-(2-aminoethyl)isothiouromium bromide (ITU), did not affect elevated colonic myeloperoxidase activity in similar rats. In addition, mice lacking a functional gene for inducible nitric oxide synthase nonetheless demonstrated elevated myeloperoxidase activity in trinitrobenzenesulfonic acid colitis, as occurs in wild-type mice with colitis (Zingarelli et al., 1999). McCafferty et al. (1997, 1999) also showed that the upregulation of inducible nitric oxide synthase was actually protective in two different colitis models (acetic acid colitis and trinitrobenzenesulfonic acid colitis). The reason why inducible nitric oxide synthase inhibition or genetically determined loss shows different effects in trinitrobenzenesulfonic acid- and dextran sulfate sodium-induced colitis is not clear. Further studies of dextran sulfate sodium-induced colitis in knockout animals or trinitrobenzenesulfonic acid-induced colitis with ONO-1714 treatment may help to resolve these issues.

Nitric oxide may also mediate tissue injury indirectly, producing peroxynitrite anion by its interaction with superoxide. Peroxynitrite modifies tyrosine residues in proteins to form nitrotyrosine, which can be detected immunohistochemically. However, a recent report demonstrated that tyrosine was nitrated most efficiently with a nitric oxide donor alone in the absence of superoxide formation, via formation of nitrogen dioxide (Pfeiffer and Mayer, 1998). Also, in the presence of hypochlorous acid or myeloperoxidase, nitrotyrosine can be formed from nitrite via formation of nitryl chloride and nitrogen dioxide (Eiserich et al., 1998). Therefore, nitrotyrosine is a marker for multiple oxidants derived from nitric oxide in vivo, not merely peroxynitrite. Formation of nitrotyrosine has been demonstrated both in clinical biopsy specimens from patients with inflammatory bowel disease (Singer et al., 1996; Kimura et al., 1998; Dijkstra et al., 1998) and in experimental models (Zingarelli et al., 1998, 1999). Our present findings that the potent inhibitor ONO-1714 reduced numbers of nitrotyrosine-immunoreactive inflammatory and epithelial cells in dextran sulfate sodium colitis supports previous observations in trinitrobenzenesulfonic acid-induced ileitis in inducible nitric oxide synthase-deficient mice (Zingarelli et al., 1999) and also observations following mercaptoethylguanidine administration in trinitrobenzenesulfonic acid-induced colitis (Zingarelli et al., 1999).

We demonstrated that neutrophil infiltration in colonic mucosa was significantly increased by dextran sulfate sodium administration over 7 days; this increase paralleled increases in lipid peroxides, suggesting that granulocytes represented an important source of reactive oxygen and nitrogen species. Nitric oxide can interact rapidly and spontaneously with molecular oxygen to yield a variety of oxidizing and nitrosating agents such as nitrogen dioxide and dinitrogen trioxide. Reactive oxygen and nitrogen species are cytotoxic agents that induce lipid peroxidation and other cellular oxidative stress by cross-linking proteins, lipids, and nucleic acids; cellular dysfunction, damage, and eventually death result. Evidence of damage by reactive species is provided by increased lipid peroxides in colonic mucosa from patients with ulcerative colitis and experimental colitis models. A recent report indicated that carbonylated protein was formed by oxidative stress in colonic mucosa from mice with dextran sulfate sodium colitis (Blackburn et al., 1999). In the present study, we found that mucosal damage induced by dextran sulfate sodium was associated with high concentrations of colonic thiobarbituric acid-reactive substances, a reliable marker for lipid peroxidation, and that this increase in lipid peroxides was significantly inhibited by ONO-1714. Our data support a previous report in which colonic thiobarbituric acid-reactive substances were markedly reduced in mice deficient in inducible nitric oxide synthase (Zingarelli et al., 1999), suggesting that overproduction of nitric oxide and reactive species derived from it plays a major role in the oxidative stress associated with inflammatory bowel disease.

Recent evidence strongly suggests that nitric oxide produced by activated inflammatory cells can act as an inter-

cellular messenger molecule capable of mediating a variety of effects including influences on cytokine production and secretion (Marcinkiewicz et al., 1995). Southey et al. (1997) also showed that amelioration of trinitrobenzenesulfonic acid colitis by a selective inducible nitric oxide synthase inhibitor, ITU, could include inhibition of nitric oxide-mediated pro-inflammatory cytokine release. In the present study, mucosal content of inflammatory cytokines including interleukin-4, interleukin-10, and interferon-γ significantly increased after dextran sulfate sodium administration, and these increases were significantly inhibited by treatment with ONO-1714. However, the effect on cytokine production or release could not be precisely evaluated because inflammatory cell infiltration was markedly inhibited by ONO-1714.

Another striking observation in the present experiments was a remarkable attenuation of the dextran sulfate sodium-induced increases in RNA expression for inducible nitric oxide synthase, indicating that nitric oxide may directly or indirectly regulate transcriptional expression of inducible nitric oxide synthase. Nuclear factor-κB is a member of a family of transcriptional factors first described in B lymphocytes that regulates transcription of inducible nitric oxide synthase. Two nitric oxide-mediated effects on the nuclear factor-κB activation pathway have been reported, a costimulatory effect and an inhibitory effect. In endothelial cells, Umansky et al. (1998) demonstrated that low concentrations of nitric oxide that could not in themselves activate gene expression by nuclear factor-κB strongly augmented nuclear factor-κB activation by tumor necrosis factor-α or phorbol myristate acetate, as shown by an in vitro kinase assay, gene expression experiments, and electrophoretic mobility shift assay. Marcinkiewicz et al. (1995) hypothesized that nitric oxide overproduction by inflammatory cells could result in activation of a positive feedback loop in which nitric oxide enhances synthesis of cytokines such as interleukin-1β and tumor necrosis factor-α, which in turn could result in increased inducible nitric oxide synthase expression. The report suggests an important self-amplifying mechanism, since inducible nitric oxide synthase is under nuclear factor-κB control. Our findings that ONO-1714 markedly attenuated expression of inducible nitric oxide synthase RNA indicated that ONO-1714 may interrupt such a positive feedback. On the other hand, increased amounts of nitric oxide result in strong inhibition of cytokine-induced nuclear factor-κB activation; Peng et al. (1998) reported that nitric oxide inhibited activation of nuclear factor-κB through induction and stabilization of the nuclear factor-κB inhibitor, IκBα. Further experiments will be needed to clarify the interactions of nitric oxide and nuclear factor-κB regulation of inducible nitric oxide synthase expression in inflamed colonic tissues.

In summary, dextran sulfate sodium-induced intestinal inflammation resulted in oxidative and nitrosative stress characterized by overproduction of nitric oxide, increased expression of inducible nitric oxide synthase, increased numbers of cells immunoreactive for nitrotyrosine, and marked accumulation of lipid peroxides. The selective inhibitor ONO-1714 acted as a remarkably effective anti-inflammatory agent in this model. Agents that inhibit inducible nitric oxide synthase activity or expression offer exciting potential as a therapeutic approach to inflammatory bowel disease.

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