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Original Research Communication

α-Phenyl-*N-tert*-Butylnitrone Provides Protection from Dextran Sulfate Sodium-Induced Colitis in Mice

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ABSTRACT

Nuclear factor-*κB* (NF-κB)-dependent up-regulation of inflammatory cytokines and inducible nitric oxide (iNOS) occurs in inflammatory bowel disease. We investigated the effect of α-phenyl*N-tert*-butylnitrone (PBN), a spin-trapping agent that inhibits NF-κB activity, on dextran sulfate sodium (DSS)-induced colonic mucosal injury and inflammation in mice. Acute colitis was induced with DSS in female BALB/c mice receiving 0, 0.3, 3, and 30 mg/kg i.p. PBN daily. Colonic mucosal inflammation was evaluated biochemically and histologically. Nitric oxide was evaluated as luminal nitrite/nitrite concentration by the Griess reaction and as immunoreactive nitrotyrosine in mucosal cells. Mucosal tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were determined by immunoassay. Colonic mRNA expression for iNOS, TNF-α, and IFN-γ was measured by reverse transcription–polymerase chain reaction, and NF-κB activation was evaluated by electrophoretic mobility shift assay. After DSS administration, mice showed increased luminal nitrite/nitrate, mucosal TNF-α and IFN-γ, and mRNA for iNOS and these cytokines, in addition to decreased colonic length and increased inflammatory score, luminal hemoglobin, and colonic myeloperoxidase activity. PBN inhibited increases in luminal nitric oxide production, nitrotyrosine immunoreactivity, and mucosal TNF-α and IFN-γ. Colonic iNOS, TNF-α, and IFN-γ mRNA were suppressed by PBN, as was a DSS-induced increase in colonic NF-κB DNA-binding activity. NF-κB is essential to DSS-induced colitis, suggesting molecular approach targeting of NF-κB for treatment of inflammatory bowel disease. Antioxid. Redox Signal. 4, 195–206.

INTRODUCTION

CROHN'S DISEASE AND ULCERATIVE COLITIS, considered together as inflammatory bowel disease, are characterized by increased expression of inflammatory cytokines, adhesion molecules, and inducible enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (13). Expression of these proteins in response to various environmental signals is regulated primarily by changes in the transcription rate of the gene controlled by sequence-specific DNA-binding proteins called transcription factors. Nuclear factor-κB (NF-κB) is a family of transcription factor first described in B lymphocytes, where they constitutively bind to and transactivate the enhancer of the immunoglobulin kappa gene. These transcription factors now are known to regulate a variety of additional genes controlling the cell cycle, immune function, and inflammatory processes (1). Further, NF-κB

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plays a pivotal role in regulating programmed cell death, having the ability to activate both proapoptotic and antiapoptotic genes. Protective effects of an antisense oligonucleotide against the p65 subunit of NF-κB in an animal model of colitis (22) suggests that blockade of this transcription factor may represent a valuable therapeutic strategy for human inflammatory bowel disease.

The dextran sulfate sodium (DSS)-colitis model used in the present experimental study has strong histologic resemblances to human ulcerative colitis, including focal crypt lesions, mucosal and submucosal inflammation, and infiltration of granulocytes (27). Recently, Herfarth *et al.* (6) demonstrated that colonic NF-κB DNA-binding activity was increased in DSS-induced colitis and that the colonic inflammation was suppressed by gliotoxin, a fungal metabolic known to inhibit NF-κB activity.

Phenyl *N-tert*-butylnitrone (PBN) is a family of nitrones used as spin-trapping agents to trap free radicals. As summarized by Kotake (11), PBN's pharmacologic effects in animal models are extensive, ranging from protection against death after endotoxin shock and protection from ischemia–reperfusion injury to increasing the lifespan of mice. Recent additions to the list include protection from bacterial meningitis, thalidomide-induced teratogenicity, drug-induced diabetogenesis, and choline-deficiency hepatocarcinogenesis. Early study suggested that PBN was a simple free radical scavenger that protected tissues from direct damage from free radical attack (8). Recently, PBN was shown to inhibit gene induction of iNOS and subsequent nitric oxide (NO) formation in an endotoxin shock model (17). More recently, PBN has shown ability to inhibit activation of transcriptional factor NF-κB in mouse macrophages stimulated with lipopolysaccharide and interferon-γ (INF-γ) (12). Because activation of NF-κB is suggested to be redox-sensitive or free radical-mediated, PBN's free radical trapping action may be involved in its inhibition of NF-κB activation. However, the effects of PBN on NF-κB activation and expression of proinflammatory cytokines after DSS administration have not been investigated. We therefore examined whether PBN can ameliorate DSS-induced intestinal inflammation in mice, and whether inhibition of NF-κB activation associated with proinflammatory cytokine expression was involved in the antiinflammatory effect.

MATERIALS AND METHODS

Chemicals

All chemicals were prepared immediately before use. PBN was a gift from Prof. Y. Kotake. Thiobarbituric acid (TBA) and 3,3′,5,5′-tetramethylbenzidine were obtained from Wako Pure Chemical (Osaka, Japan), and 1,1,3,3-tetramethoxypropane was obtained from Tokyo Kasei (Tokyo, Japan). Enzyme-linked immunosorbent assay kits for tumor necrosis factor-α (TNF-α), and IFN-γ were obtained from BioSource International (Camarillo, CA, U.S.A.). All other chemicals used were of reagent grade.

Animals

Nine-week-old female BALB/c mice weighing 18–20 g were obtained from Keari (Osaka, Japan). Mice were housed in our animal quarters prior to experiments and were maintained at 18–24°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 the colon was removed.

Induction of colitis and design of treatment

Acute colitis was induced by giving 8% DSS (molecular weight, 8,000; lot no. DS-605, Seikagaku, Tokyo, Japan) orally in drinking water for 7 days. PBN was dissolved in physiologic saline. Mice were randomized into groups receiving different concentrations of PBN (0.3, 3, and 30 mg/kg/day, or only physiologic saline) by intraperitoneal injection on each of the 7 days. Mice were killed on day 7, and colons were removed for histologic examination. Colonic specimens also

were taken for biochemical assay and RNA isolation.

Evaluation of colonic inflammation

Colonic inflammation was evaluated by body weight, total colon length, luminal hemoglobin, and histologic score. Colonic bleeding was qualified indirectly as hemoglobin concentration in luminal lavage fluid using a kit according to the manufacturer's protocol (Wako). For histologic evaluation, formalin-fixed tissues were stained with hematoxylin and eosin and evaluated by light microscopy by a pathologist unaware of the experimental conditions for any given specimen. Histologic features of inflammation were assessed by a semiquantitative scoring system adapted to the murine model (2).

As an index of neutrophil accumulation, tissue-associated myeloperoxidase (MPO) activity was determined by a modification of the method of Grisham *et al.* (4). Two milliliters of mucosal homogenate was centrifuged at 20,000 *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 *g* for 15 min at 4°C, and supernatants were saved. MPO 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 MPO that caused a change in absorbance of 1.0/min at 655 nm and 25°C.

Measurement of TBA-reactive substances

The concentration of TBA-reactive substances was measured in the colon mucosa using the method of Ohkawa *et al.* (26) as an index of lipid peroxidation. Animals were killed by exsanguination from the abdominal aorta after experimental treatments, colons were removed, and the mucosa was scraped off using two glass slides. The mucosa then was homogenized with 1.5 ml of 10 m*M* potassium phosphate buffer (pH 7.8) containing 30 m*M* KCl in a Teflon Potter–Elvehjem homogenizer. The amount of TBA-reactive substances in mucosal homogenates was exposed as nanomoles of malondialdehyde per milligram of protein using 1,1,3,3-tetramethoxypropane as the standard. Total protein in the tissue homogenates was measured by the method of Lowry *et al.* (14).

Nitrate/nitrite assay

NO production was quantified indirectly as nitrite plus nitrate concentration in luminal lavage fluid. Luminal lavage fluid was centrifuged, and the supernatant was divided into aliquots and frozen until assay. 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 using a kit according to the manufacturer's protocol (Dojin, Kumamoto, Japan). Nitrite concentrations were calculated by comparison with standard solutions of sodium nitrate prepared in saline solution after reduction.

Immunohistochemistry for nitrotyrosine

Tyrosine nitration was evaluated in colonic sections by immunohistochemistry with a kit according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, U.S.A.). Paraffin-embedded colonic specimens were sectioned, deparaffinized, and rehydrated. Sections then were placed in a Coplin jar for antigen retrieval in 10 m*M* 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 2–3 min. Rinsed slides were treated with 2% H_2O_2 for 5 min to block endogenous peroxidase activity, and then were rinsed briefly in phosphate-buffered saline. Nonspecific binding was blocked by incubating the slides with phosphate-buffered saline containing 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.

Determination of colonic content of inflammatory cytokines

Concentrations of proinflammatory cytokines (TNF-α and IFN-γ) in the supernatant of mucosal homogenates were determined using enzyme-linked immunosorbent assay kits with antibodies specific for the respective mouse cytokines.

Reversed transcription–polymerase chain reaction (RT-PCR) for iNOS and cytokine message

Samples for mRNA 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. RNA was stored at −70°C until RT was performed. One microliter of RT product was added to 3 m*M* concentrations of primers for iNOS, TNF-α, IFN-γ, 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 iNOS, sense 5′-TCACTGGGACAGCACAGAAT-3′, and antisense 5′-TGTGTCTGCAGATGTGCTGA-3′; for IFN-γ, sense 5′-TACTGCCACGGCACAGTCATTGAA-3′, and antisense 5′-GCAGCGACTCCTTTTCCGCTTCCT-3′; for TNF-α, sense 5′-ATGAGCACAGAAAGCATGATC-3′, and antisense 5′-TACAGGCTTGTCACTCGAATT-3′; and for β-actin, sense 5′-TGTGATGGTGGGAATGGGTCAG-3′, and antisense 5′-TTTGATGTCACGCACGATTTCC-3′. The mixture was subjected to PCR 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.

Electrophoretic mobility shift assay (EMSA)

Binding activity of NF-κB to its consensus oligonucleotide was analyzed in nuclear extracts of mouse colonic tissue cells by EMSA using a nonradioactive EMSA kit DIG gel-shift kit (Boehringer–Mannheim, Mannheim, Germany). NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was end-labeled with digoxigenin (DIG). Nuclear extracts (14 μg) were added to DIG-labeled NF-κB oligonucleotide (25 pmol) in a buffer containing 1 μg of poly[d(I-C)], poly-L-lysine, 20 m*M* HEPES (pH 7.6), 1 m*M* EDTA, 10 m*M* $(NH_4)_2SO_4$, 1 m*M* dithiothreitol, and 30 m*M* KCl (total volume, 20 μl). This solution was subjected to electrophoresis on a 5% polyacrylamide gel followed by electrotransfer to a nylon membrane. The membrane then was treated with anti-DIG antibody, and then with alkaline phosphatase-conjugated IgG. A chemiluminescent substrate (CDP-star; Amersham Pharmacia Biotech, Buckinghamshire, U.K.) was added, and resulting chemiluminescence was detected by autoradiography.

Statistics

The results are presented as the mean + SEM. Data were compared by one-way analy-

TABLE 1. EFFECTS OF PBN ON BODY WEIGHT, TOTAL LENGTH OF THE COLON, AND INTRALUMINAL HEMOGLOBIN IN DSS-INDUCED COLITIS IN MICE

	Body weight gain (g/7 days)	*Total length of the colon (cm)*	*Luminal hemoglobin (mg/cm)*
No colitis	0.28 ± 0.17	11.43 ± 0.14	0.80 ± 0.19
DSS only	−2.42 ± 0.21*	9.10 ± 0.37*	6.29 ± 1.12†
PBN plus DSS			
0.3 mg/kg	−2.12 ± 0.46‡	9.80 ± 0.26‡	3.08 ± 1.21‡
3.0 mg/kg	−1.34 ± 0.19‡	10.25 ± 0.43‡	2.21 ± 0.41‡
30 mg/kg	−0.96 ± 0.21§	10.30 ± 0.20§	3.29 ± 0.73‡

Each value indicates the mean ± SEM for five mice. PBN dissolved in physiologic saline was administered to mice by intraperitoneal injection.

*$p < 0.05$ and †$p < 0.01$, compared with mice without colitis.

‡$p < 0.05$ and §$p < 0.01$, compared with mice receiving only DSS.

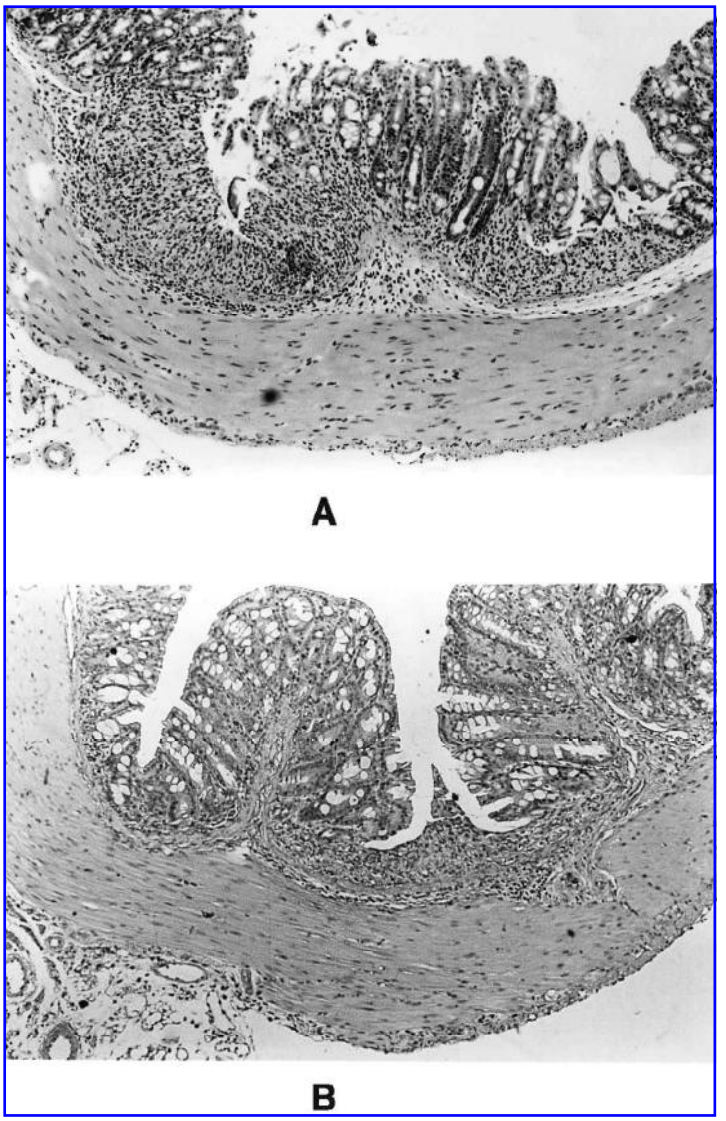

FIG. 1. Appearance of the colon in a DSS-treated mouse (A) and a mouse also receiving 3 mg/kg PBN (B). Loss and shortening of crypts, mucosal erosions, inflammatory cell infiltration, and goblet cell depletion are seen in A. In B, smaller erosions are associated with less inflammatory cell infiltration. Hematoxylin and eosin, ×20.

sis of variance. Differences were considered significant if the p value was <0.05 based on Dunnett's multiple comparison test. All analyses were performed using the Stat View 5.0-J program (Abacus Concepts, Berkeley, CA, U.S.A.) with a Macintosh computer.

Ethical considerations

Maintenance of animals and experimental procedures were carried out in accordance with the U.S. 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).

RESULTS

Effect of PBN on experimental colitis

Mice exposed to 8% DSS developed symptoms of acute colitis, with diarrhea being observed first, followed by rectal bleeding and severe weight loss. Shortening of the colon was observed upon removal. Decreases in body weight and total colon length were significantly reversed by treatment with PBN at doses of 0.3, 3.0, and 30 mg/kg (Table 1). Lavage fluid hemoglobin concentrations showed approximately eightfold elevations in untreated control mice with colitis. This elevation was abolished by PBN treatment.

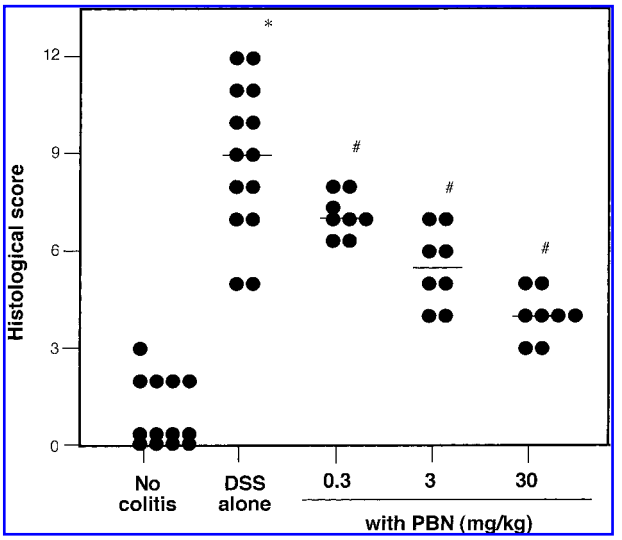

FIG. 2. Effect of PBN on histologic score in colonic mucosa of mice administered 8% DSS. *p < 0.01, compared with mice without colitis; $p < 0.05$, compared with mice receiving only DSS.

The protective effect of PBN was confirmed by histologic study. Figure 1 shows typical histologic appearance in a PBN-treated group (3.0 mg/kg) and controls. Administration of 8% DSS alone for 7 days resulted in large areas of epithelial crypt loss, prominent neutrophilic infiltration throughout the mucosa, ulceration, and mucosal bleeding. In contrast, treatment with PBN resulted in smaller erosions with few neutrophils. Administration of PBN significantly reduced the histologic score compared with findings in DSS-exposed control mice (Fig. 2).

Neutrophil accumulation also was evaluated by measurement of MPO activity in colonic mucosal homogenates. MPO activity increased significantly from a basal mean concentration of 0.21 ± 0.04 mU/mg of protein to 1.34 ± 0.33 mU/mg of protein at 7 days after DSS administration (Fig. 3). The increase in MPO activity in colonic mucosa with DSS administration was significantly inhibited in a dose-dependent manner by treatment with PBN.

Effect of PBN on TBA-reactive substances in colonic mucosa

TBA-reactive substances in colonic mucosa increased significantly from a basal mean concentration of 0.739 ± 0.056 nmol/mg of protein to 1.482 ± 0.125 nmol/mg of protein at 7 days after DSS administration (Fig. 4). The increase in TBA-reactive substances was significantly inhibited by treatment with PBN at doses of 0.3, 3.0, and 30 mg/kg (Fig. 4).

Effect of PBN on luminal nitrite/nitrate concentration and nitrotyrosine formation in the colonic tissue

To test whether PBN inhibited NO production *in vivo*, we measured luminal concentrations of nitrite plus nitrate by the Griess reaction, and formation of nitrolyrosine by immunohistochemical study of colonic tissue section. Nitrite plus nitrate concentrations in the colonic lumen of mice with colitis were significantly elevated (approximately fivefold) beyond concentrations in control mice receiving no DSS. These increases were largely abolished by PBN treatment at doses of 0.3, 3.0, and 30 mg/kg (Table 2).

To evaluate peroxynitrite formation in the present model, we performed immunohistochemistry for nitrotyrosine. Immunoreactivity for nitrotyrosine was evident in cells throughout the gut wall in DSS-induced colitis, being particularly strong in epithelial cells, neutrophils, and macrophages (Fig. 5). No staining was evident when the antibody was preincubated with the antigen, nitrotyrosine. Coadministration of PBN to mice greatly prevented enhanced immunohistochemical staining for nitrotyrosine.

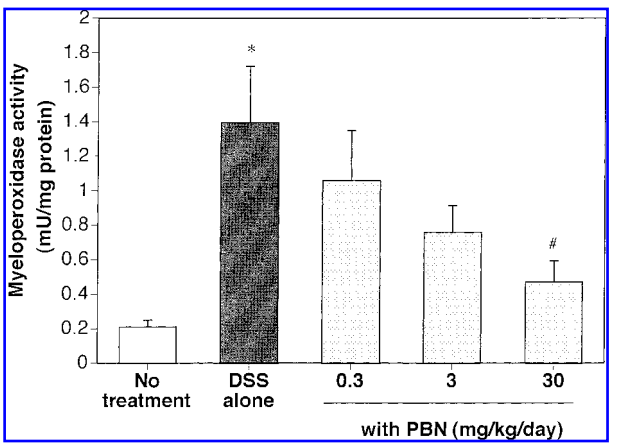

FIG. 3. Effect of PBN on neutrophil accumulation expressed as MPO activity in colonic mucosa of mice administered 8% DSS. Values represent the means ± SEM for six to eight mice. *$p < 0.05$, compared with untreated mice; #$p < 0.05$, compared with mice receiving only DSS.

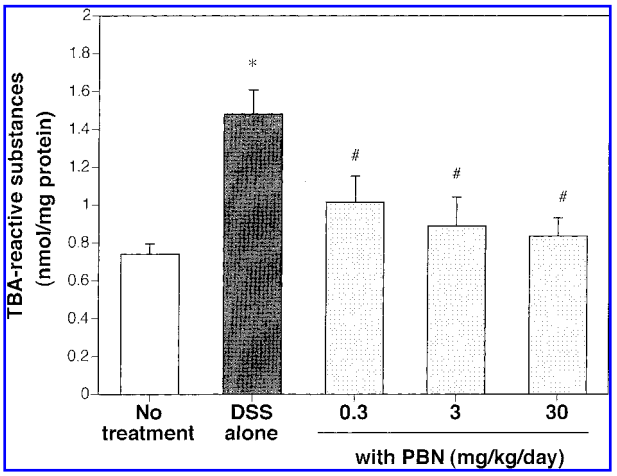

FIG. 4. Effect of PBN on the increase in lipid peroxide concentration in colonic mucosa of mice administered 8% DSS. Values represent the means ± SEM for six to eight mice. *$p < 0.01$, compared with untreated mice; #$p < 0.01$, compared with mice receiving only DSS.

Effect of PBN on mucosal content of inflammatory cytokines

The colonic content of inflammatory cytokines (TNF-α and IFN-γ) was increased significantly in 7 days after DSS administration (Table 2). Increases in inflammatory cytokines in colonic mucosa were significantly inhibited by PBN at doses of 0.3, 3, and 30 mg/kg (Table 2).

Effect of PBN on mRNA expression of iNOS and inflammatory cytokine genes

To analyze further the effects of PBN on experimental intestinal inflammation, we assessed colonic mRNA expression for iNOS, TNF-α, and IFN-γ using semiquantitative RT-PCR yielding 456-, 276-, 405-, and 514-bp products to identify iNOS, TNF-α, INF-γ, and β-actin gene expression respectively. As shown in Fig. 6, we found iNOS and TNF-α gene expression in control animals to be negligible or faint. In contrast, transcription was readily shown in DSS-treated mice throughout the time course of the study, with peak expression on day 7. Expression of IFN-γ was present in mice before DSS administration, and these were increased after DSS administration. Treatment with PBN suppressed mRNA expression for each gene.

Effect of PBN on NF-κB DNA-binding activity

The administration of DSS alone enhanced NF-κB DNA-binding activity in inflamed colonic tissue; enhancement was suppressed by PBN administration at a dose of 3.0 mg/kg (Fig. 7).

DISCUSSION

The present study demonstrates for the first time that PBN, a spin-trapping agent, attenuates DSS-induced colonic injury and inflammation in mice. In our study, intestinal injury was assessed by a variety of methods, including body weight, length of the colon, luminal content of hemoglobin, granulocytic infiltration, and histology. By each assessment, PBN treatment significantly inhibited colonic injury. In addition, we showed that, in DSS-induced intestinal inflammation, NF-κB DNA-binding activity was enhanced in asso-

TABLE 2. EFFECTS OF PBN ON LUMINAL CONTENTS OF NITRITE/NITRATE AND MUCOSAL CONTENTS OF INFLAMMATORY CYTOKINES DSS-INDUCED COLITIS IN MICE

	Nitrite/Nitrate (nM/cm)	*TFN-α* (pg/mg of protein)	*INF-γ* (pg/mg of protein)
No colitis	0.83 ± 0.08	7.52 ± 0.80	27.9 ± 6.2
DSS only	3.94 ± 0.64*	10.86 ± 2.10*	64.6 ± 14.7†
PBN			
0.3 mg/kg	1.84 ± 0.21‡	4.86 ± 0.60‡	26.9 ± 3.9‡
3.0 mg/kg	1.56 ± 0.25‡	6.68 ± 1.21‡	37.0 ± 5.3‡
30 mg/kg	1.92 ± 0.27‡	5.95 ± 0.80‡	37.8 ± 6.2‡

Each value indicates the mean ± SEM for five mice. PBN dissolved in physiologic saline was administered to mice by intraperitoneal injection.

*$p < 0.05$ and †$p < 0.01$, compared with mice without colitis.

‡$p < 0.05$, compared with mice receiving only DSS.

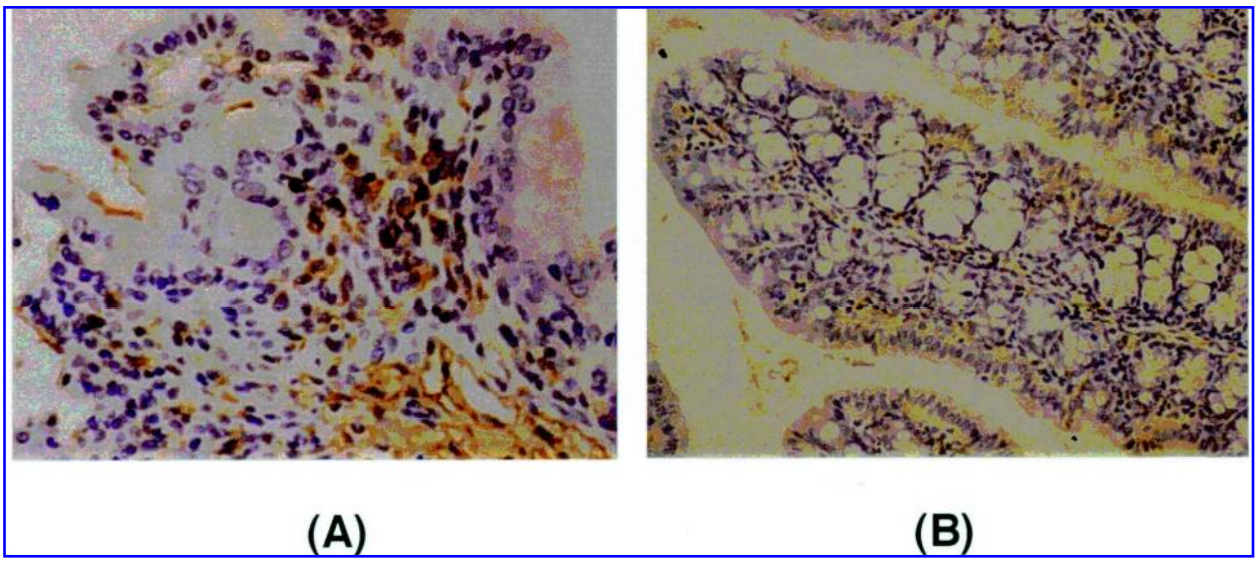

FIG. 5. Immunohistochemical detection of nitrotyrosine in inflamed colon from a mouse treated with only DSS (A) and a mouse also receiving PBN (B). In A, extensive immunoreactivity is noted throughout the villus including epithelial cells, inflammatory cells, and interstitial tissues. In B, PBN cotreatment has greatly reduced immunohistochemical staining for nitrotyrosine. Magnification, $\times 100$.

ciation with increased mRNA expression for iNOS, IFN-γ, and TNF-α, which are known to have κB binding sites in their promoter or enhancer regions. We also demonstrated that PBN treatment inhibited up-regulation of expression of these genes by interfering with NF-κB activation *in vivo.*

Although histological score and MPO activity inhibition show dose-dependent response to PBN, tests of PBN high doses (3.0 and 30 mg/kg) exhibited confusing results on total length of the colon and luminal hemoglobin, as well as on nitrate/nitrite luminal and inflammatory cytokine mucosal contents in mice. These results are indicative of multiple radical scavenging by PBN and raise questions about its specificity. To find specificity of its effect, it appears that it will be more logical to test PBN concentrations lower than 0.3 mg/kg. In the preliminary dose–response study on the pro-

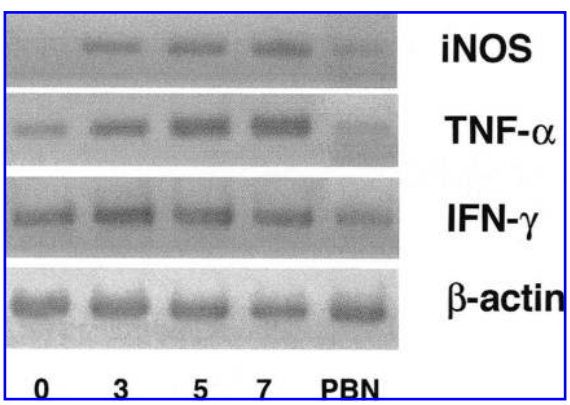

FIG. 6. Effects of PBN on mRNA expression for iNOS, TNF-α, and IFN-γ in colonic tissues of mice given 8% DSS. A representative 2% agarose gel of RT-PCR products is shown, also including β-actin mRNA, for normal colon tissue (0), for inflamed tissue at 3 days (3), 5 days (5), and 7 days (7), and for inflamed tissue cotreated with PBN at a dose of 3.0 mg/kg at 7 days.

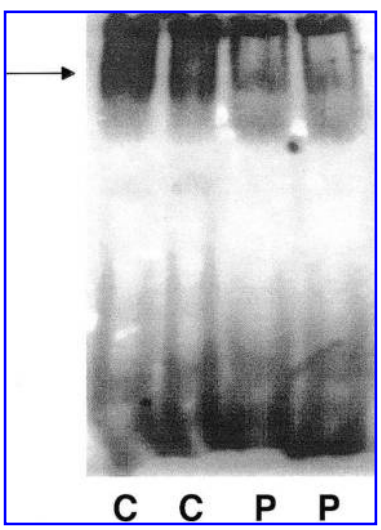

FIG. 7. Effects of DSS (lanes C) and PBN (lanes P) on NF-κB DNA-binding activity.

tective action of PBN at doses from 0.01 to 100 mg/kg, we confirmed that PBN inhibited the decrease of colon length with 50% inhibition at 1.5 mg/kg (data not shown). In addition, the inhibitory action against NF-κB activation was reported to be obtained by the use of millimolar concentrations of PBN *in vitro* (12). Judging from these data, we set the doses of PBN at 0.3, 3.0, and 30 mg/kg and tested it.

An important observation of the present study is that TBA-reactive substances, an index of lipid peroxidation, are significantly increased in the colonic mucosa after DSS administration; this increase is significantly inhibited by treatment with PBN at doses of 0.3, 3, and 30 mg/kg. Compared with results on histological score and MPO, the PBN effect on lipid peroxidation in colon is less impressive. As one reason, it is thought that the inhibitory activity of PBN against lipid peroxidation is relatively weak as compared with hydrophobic phenolic antioxidants (32). Although the TBA test is not specific for lipid peroxides, it is one of the oldest and most frequently used methods for measuring the peroxidation of fatty acid, cell membranes, and food products, and it can be applied to crude biological sample. By measuring the TBA-reactive substances, we have reported that lipid peroxidation mediated by oxygen free radicals is involved in the gastrointestinal mucosal injuries induced in animal models by various types of stress (33, 34), ischemia–reperfusion (35), or inflammation (7, 36). Polyunsaturated fatty acids of cell membranes are degraded by lipid peroxidation with subsequent disruption of membrane integrity, suggesting that lipid peroxidation mediated by oxygen radicals is an important cause of damage and destruction of cell membranes (23, 24). Our data support another recent finding in our laboratory, in which colonic TBA-reactive substances were markedly reduced in the same DSS colitis mouse model by treatment with a synthetic vitamin E analogue (unpublished observation); this suggests that induction of lipid peroxidation is an early critical event in this experimental inflammatory bowel disease.

Up-regulation of iNOS expression and overproduction of NO have been observed in inflamed colonic tissue from patients with inflammatory bowel disease (15, 31), trinitrobenzenesulfonic acid (TNBS)-treated guinea pigs (16), and IL-2-deficient mice (5). Formation of nitrotyrosine, a marker of oxidants derived *in vivo* from NO, also has been demonstrated both in clinical biopsy specimens from patients with inflammatory bowel disease (3, 9, 31) and in experimental models (37, 38). In the present study, increased expression of iNOS mRNA coincided with increased luminal NO production, as well enhanced immunoreactivity for nitrotyrosine in the inflamed colonic wall; up-regulation of the NO pathway was inhibited by PBN treatment. Reduction of NO formation by PBN was confirmed by an NO-trapping study in which PBN pretreatment 30 min before lipopolysaccharide injection was shown to inhibit NO formation in the liver in a mouse model of shock (17). This reduction has been shown to result from inhibition of iNOS gene and protein expression (18). More recently, we demonstrated the protective effect of a novel specific iNOS inhibitor in DSS-induced colitis in mice (21). These findings suggest that induction of iNOS is a crucial event in this model of inflammatory bowel disease, and that part of PBN's antiinflammatory action is derived from interaction with NO pathways.

Recent various experimental murine models of inflammatory bowel disease support a central role for T cells in chronic intestinal inflammation, in which an imbalance of regulatory cytokines, most notably an excessive production of TNF-α and IFN-γ, is apparent. Previous reports of DSS colitis in mice have emphasized involvement of T-helper 1 responses with production of TNF-α and IFN-γ in this model of colitis; treatment with anti-TNF-α antibody or anti-IFN-γ antibody markedly decreased severity (10, 25). We also examined cytokine expression in the inflamed colonic tissues of DSS-treated mice. Expression of mRNA encoding TNF-α and IFN-γ was increased over that in a control group not exposed to DSS, an observation consistent with elevated binding activity of NF-κB in nuclear extracts of mouse colonic cells to its consensus oligonucleotide that was demonstrated by EMSA. Our previous study

demonstrated that the mRNA expression for iNOS, TNF-α, and IFN-γ was increased gradually after DSS administration with a peak by the seventh-day, which was consistent with the elevated NF-κB activation (20). Therefore, we tested the effects of PBN on NF-κB activation as well as inflammatory responses on day 7 after the induction of colitis. The importance of NF-κB transcription factors in inflammatory bowel disease is supported by recent experiments where antisense phosphorothionate oligonucleotides corresponding to the p65 subunit of NF-κB abrogate colitis in IL-10-deficient mice. TNBS-treated mice (22), and DSS-treated mice (19). To investigate further the immunoregulatory and pathophysiologic role of NF-κB in intestinal inflammation, we coadministered PBN intraperitoneally with DSS. Recently, Kotake *et al.* (12) suggested that the previously described anti-inflammatory action of PBN most likely involved an ability to specifically inhibit NF-κB activation. In the present study, we confirmed their findings *in vivo* by demonstrating down-regulation of NF-κB activation, as well as expression of the κB-inducible genes TNF-α, INF-γ, and iNOS in inflamed mucosa. Our data are in agreement with a recently reported study in which PBN inhibited NF-κB and expressions of TNF-α and IL-1β *in vivo* in a rat endotoxin shock model (29). Free radicals have been proposed to act as signaling molecules activating kinases that in turn activate IκB kinase, which can phosphorylate IκB. Although PBN deactivates free radicals, the precise mechanism in which PBN inhibits NF-κB activation is not completely resolved. Further studies will be required to clarify fully the mechanism of PBN-induced reduction of inflammation.

In conclusion, our results showed that DSS-induced intestinal inflammation is characterized by increased NF-κB DNA-binding activity. Blockade of NF-κB by PBN was accompanied by significant suppression of intestinal inflammation and mRNA expression of TNF-α, IFN-γ, and iNOS *in vivo*. Together with the reported observations of NF-κB activation in patients with inflammatory bowel disease (28, 30), these data suggest that therapeutic targeting of the NF-κB/IκB system represents a promising novel molecular approach to reducing intestinal inflammation.

ABBREVIATIONS

DIG, digoxigenin; DSS, dextran sulfate sodium; EMSA, electrophoretic mobility shift assay; IFN-γ, interferon-γ; IL, interleukin; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; NO, nitric oxide; PBN, α-phenyl-*N-tert*-butylnitrone; RT-PCR, reversed transcription–polymerase chain reaction; TBA, thiobarbituric acid; TNBS, trinitrobenzenesulfonic acid; TFN-α, tumor necrosis factor-α.

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