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Original Contribution

EVIDENCE FOR OXIDATIVE STRESS IN NSAID-INDUCED COLITIS IN IL10$^{-/-}$ MICE

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Abstract—The goal of this study was to evaluate for evidence of oxidative stress in colonic inflammation in a novel model of inflammatory bowel disease, nonsteroidal anti-inflammatory drug- (NSAID-) treated interleukin-10-deficient (IL10$^{-/-}$) mice. IL10$^{-/-}$ and wild-type (wt) mice were treated with a nonselective NSAID (piroxicam, 200 ppm in the diet) for 2 weeks to induce colitis, and parameters for oxidative stress in the colonic tissues were evaluated. Mean chemiluminescence enhanced with lucigenin in the colons from IL10$^{-/-}$ mice treated with piroxicam was more than 5-fold higher than that of the control wt group. Chemiluminescence was inhibited with diphenylethylene iodinium, but not allopurinol, indomethacin, or N-ω-nitro-L-arginine, indicating that flavin-containing enzymes were the source of the reactive oxygen species. Colonic aconitase activity in NSAID-treated IL10$^{-/-}$ mice decreased to 50% of the activity of control mice. There was no difference in the total glutathione levels in the colonic mucosa among the groups; however, glutathione disulfide levels were approximately 2-fold greater in the colon of NSAID-treated IL10$^{-/-}$ mice as compared with control groups. Immunohistochemistry studies of colons from NSAID-treated IL10$^{-/-}$ mice demonstrated intense staining with two antibodies that recognize advanced glycation endproducts formed through glycation and oxidation: anticarboxymethylysine and antipentosidine. The epithelial cells and lamina propria cells in the colons of NSAID-treated IL10$^{-/-}$ mice showed immunostaining with antinitrotyrosine, indicating the presence of reactive nitrogen species. Colonic epithelium of IL10$^{-/-}$ mice with colitis showed moderate immunostaining for 8-hydroxy-2'-deoxyguanosine in the nuclei. NSAID-treated IL10$^{-/-}$ mice treated with diphenylene idodonium chloride (DPI), an irreversible inhibitor of flavoprotein enzymes, experienced significantly reduced inflammation. Taken together, these results strongly indicate the presence of oxidative stress in the inflammatory bowel disease in NSAID-treated IL10$^{-/-}$ mice and suggests a role for oxidative stress in the pathophysiology of this model of inflammatory bowel disease.

Keywords—Inflammatory bowel disease, Interleukin-10, Nonsteroidal anti-inflammatory drug, Free radicals, Reactive oxygen species, Reactive nitrogen species

INTRODUCTION

Oxidative stress may play an important role in the pathophysiology of inflammatory bowel disease (IBD). The colons of individuals with IBD are infiltrated with neutrophils and activated macrophages that are capable of producing high levels of reactive oxygen and nitrogen species. In addition, inflammatory cytokines such as TNF-α and IFN-γ, which are overproduced in IBD, are potent inducers of reactive oxygen species (ROS) and NO. Increased luminol or lucigenin-amplified chemiluminescence indicating the presence of reactive oxygen species have been reported in colon biopsies from individuals with IBD [1,2]. Additionally, increased nitrotyrosine levels, which indicate increased levels of reactive nitrogen species [3], have been documented in colonic

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biopsies from individuals with IBD, further suggesting a role for oxidative stress in this disease. Reactive oxygen and nitrogen species are highly reactive in vivo and excessive production can lead to tissue damage of the host via oxidation of lipids, proteins, and DNA. Moreover, chronic inflammatory bowel diseases, both ulcerative colitis and Crohn's disease, are significant risk factors for the development of colon cancer [4,5]. Although the mechanisms by which colitis promotes the development of colon cancer are not fully understood, inflammation-induced oxidants may be carcinogenic since they can induce mutations in proto-oncogenes and tumor suppressor genes and, thus, promote the development of colon cancer.

Interleukin-10 (IL10) is a cytokine with potent anti-inflammatory and immune regulatory activity. IL10 inhibits the production of inflammatory cytokines, such as IL1 and TNF-α, which stimulate production of reactive oxygen species [6]. IL10 also inhibits production of reactive oxygen species in neutrophils and human monocytes [7,8]. We have reported previously that IL10-deficient mice ($IL10^{-/-}$) develop a spontaneous inflammatory bowel disease 3–6 months after birth [9]. This enterocolitic inflammation is associated with uncontrolled cytokine production by activated macrophages as well as IFN-γ production from $CD4^+$ Th1-like T cells. Anti-IFN-γ antibody (Ab) or anti-IL12 treatment [10] significantly attenuated intestinal inflammation in young $IL10^{-/-}$ mice, further suggesting that a pathogenic Th1 response plays a major role for the inflammation [9,10]. Moreover, T cell transfer studies demonstrate that IBD in $IL10^{-/-}$ mice is T cell dependent [11]. The $IL10^{-/-}$ model is considered to be a well-characterized model that has many features similar to human IBD. Indeed, based on this model there have been several clinical trials using IL10 treatment for IBD in human patients [12].

Recently, we reported that treatment with piroxicam, a nonselective NSAID, rapidly induces IBD in $IL10^{-/-}$ mice [13] and that NSAID treatment increases parameters indicative of oxidative stress in HepG2 cells [14]. This mimics what can be seen in humans with IBD, as it is well known that NSAID treatment can exacerbate IBD and reactivate quiescent disease [15,16]. NSAID-induced colitis in $IL10^{-/-}$ mice is characterized by infiltration of the colon with $CD4^+$ T cells and macrophages, increased inflammatory cytokine production and IFN-γ production, and colonic epithelial proliferation, all of which are similar to those of spontaneous colitis in $IL10^{-/-}$ mice. NSAID-induced colitis has a great advantage over the spontaneous IBD model in that the time required for development of colitis is substantially shortened and the variability between animals is significantly reduced, making this a much more useful model for the study of the mechanisms underlying the pathophysiology of the inflammatory bowel disease.

There are numerous reports suggesting a role for oxidative stress in animal models of IBD [17–19]. However, most of the animal studies of oxidative stress in the development of colitis have used acute disease models that may not necessarily reflect T cell-mediated IBD. The goal of this study was to determine the extent to which IBD in NSAID-induced colitis in $IL10^{-/-}$ mice is accompanied by oxidative stress.

MATERIALS AND METHODS

Animals

Healthy 4 to 5 week old $IL10^{-/-}$mice generated on a 129/SvEv background [9] and maintained in our animal care facility were used for the study. Wild-type (wt) 129/SvEv mice were obtained from Taconic Farms (Germantown, NY, USA). Mice were maintained in microisolator cages under specific pathogen-free conditions at the animal care facility at the University of Iowa. Research was conducted according to the principles of the Institutional Animal Care and Use Committee of the University of Iowa.

Induction of colitis

The rapid induction of colitis in $IL10^{-/-}$ mice by nonsteroidal anti-inflammatory drug was described previously [13]. Both wt and $IL10^{-/-}$ mice were fed either control diet (NIH-31M) or diet supplemented with piroxicam (4-hydroxy-2-methyl-3-[pyrid-2-yl-carbamoyl]-2H-1,2-benzothiazine 1,1-dioxide; Sigma Chemical Co., St. Louis, MO, USA) at a dose of 200 ppm for 2 weeks. Mice that received piroxicam for the 2 week feeding period are referred to as having acute colitis. In some experiments, after the 2 week piroxicam treatment, the mice were subsequently placed on standard rodent chow for 4 to 5 weeks. Prior studies [13] demonstrate that colitis persists at the same intensity after discontinuation of NSAID. Animals treated in this manner are referred to as having chronic colitis.

Histopathology

Two weeks after being fed piroxicam, mice were euthanized and colon specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, and 6 μm sections were stained with hematoxylin and eosin.

Chemiluminescence response of colonic tissue

The proximal 2 cm of mouse colon, not including the cecum, was removed and rinsed with ice-cold PBS, opened, and longitudinally cut into two strips. One of the strips was used for chemiluminescence assay and the

other half was immediately frozen in dry ice and stored at −80°C for the measurement of aconitase activity. The colonic tissue strips were transferred to 5 μM lucigenin immediately prior to assessment of the chemiluminescence response [20]. The chemiluminescence was measured in Femtomaster FB12 (Zylux Corp., Maryville, TN, USA). Sample photon emission was measured for 5 min, after 1.5 min dark adaptation. Subsequently, 10 μM (final concentration) of NADPH dissolved in PBS was added to the vial and chemiluminescence was measured.

To assess the source of the reactive oxygen species, colonic tissue was incubated in the presence of inhibitors including: diphenileneiodonium chloride (DPI, 100 μM), allopurinol (100 μM), indomethacin (100 μM), or N-ω-nitro-L-arginine (L-NA, 100 μM). Indomethacin and L-NA were dissolved in PBS. Allopurinol was dissolved in 1 N NaOH in high concentration and diluted in PBS. The final pH of the solution was confirmed to be 7.0. DPI was dissolved in DMSO and further diluted in PBS. Preincubation with DMSO at the same concentration as in the DPI solution did not change colonic tissue chemiluminescence (data not shown). The proximal colon from five NSAID-treated IL10$^{-/-}$ mice was divided into five strips and each strip was preincubated with the reagents or vehicle, and the lucigenin-amplified signal was measured. The doses of the inhibitors were based on previous studies [1,2,20–22]. The colonic tissue strips were freeze-dried and lucigenin-amplified chemiluminescence is expressed as the number of photons/s/mg dry weight of tissue after subtraction of the background count. The blank value (chemiluminescence without tissue) was subtracted from each value. In some studies, lucigenin-enhanced chemiluminescence with colonic strips was also measured in the presence of either Cu,Zn superoxide dismutase (SOD) from bovine erythrocytes (Oxis International, Portland, OR, USA) at a final concentration of 1000 U/ml or catalase (Sigma Chemical Co.) at 1000 U/ml.

Aconitase activity

Frozen colonic tissue was thawed in 1000 μl of buffer (50 mM Tris-HCl, pH 7.4, 0.5 mM $MnCl_2$, 0.2 mM sodium citrate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 100 μg/ml PMSF) and homogenized on ice. After centrifugation at 1000 × *g* for 10 min at 4°C, supernatants were collected for measurement of aconitase activity. Tissue aconitase activity was measured spectrophotometrically either by direct assay of measuring *cis*-aconitate absorbance decrease at 240 nm [23,24] or by coupled assay with isocitrate dehydrogenase, in which NADP reduction is monitored [25]. The former assay was performed in 50 mM Tris HCl (pH 7.4) containing 20 mM DL-trisodium isocitrate. An extinction coefficient for *cis*-aconitate of 3.6 mM^{-1} at 240 nm was used. The latter assay was performed with a commercial kit (Bioxytech Aconitase-340, Oxis International) according to the manufacturer's instructions. Protein concentration was measured using a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA), based on the method of Bradford, and activity was expressed as mU (nmol/min)/mg protein.

Measurement of glutathione content

The proximal 2 cm portion of the colon (excluding the cecum) was flushed with ice-cold PBS to remove fecal material, cut longitudinally, and opened flat. The mucosa was scraped with glass slides and immediately put into 5% (w/v) 5-sulfosalicylic acid solution. Colonic mucosa was then homogenized with pestle and centrifuged 10,000 × *g* for 5 min at 4°C. The supernatant was used for glutathione assay. Total glutathione content was determined by the method of Anderson [26]. Glutathione (GSH) and glutathione disulfide (GSSG) were distinguished by the addition of 4 μl of a 1:1 mixture of 2-vinylpyridine and ethanol per 20 μl of sample, followed by incubation for 2.5 h and assay as described previously by Griffith [27]. All glutathione determinations were normalized to the precipitated tissue proteins of the acid-treated mucosa samples. Protein pellets were resuspended in 5% (w/v) SDS in 0.1 M NaOH, and the concentrations were measured using the method of bicinchoninic acid protein assay with the Micro BCA protein assay reagent kit (Pierce Biotechnology, Inc., Rockford, IL, USA), with bovine serum albumin as standard.

Immunohistochemical detection of biomarkers related to oxidative stress

N^{ϵ}-carboxymethyllysine (CML), pentosidine, and nitrotyrosine. Colonic tissue from mice after 2 weeks of NSAID treatment were analyzed for the immunohistochemical detection of markers of oxidative stress. To determine whether biomarkers of oxidative stress would be altered in chronic colitis, after the 2 week piroxicam treatment the mice were subsequently placed on standard rodent chow for 4 to 5 weeks. Colonic tissue with chronic colitis was subsequently assessed for CML and pentosidine staining.

Colonic specimens were fixed in 10% neutral-buffered formalin, routinely processed, and sectioned at 6 μm. After deparaffinization and rehydration, antigen retrieval was performed for detection of 3-nitrotyrosine by heating the glass slides in 10 mM citrate buffer, pH 6.0, with a microwave oven at 500 W for 10 min. After cooling to room temperature, the sections were blocked with endogenous peroxidases by incubating sections with 0.3% hydrogen peroxide for 30 min. Specimens were subsequently incubated with 1% BSA for 60 min

followed by 5% normal goat serum and 1% BSA for 60 min. Slides were incubated with primary antibody overnight at 4°C. Primary antibodies include: polyclonal antinitrotyrosine antibody raised in rabbit (1 μl/ml) supplied by Upstate Biotechnology (Lake Placid, NY, USA), purified antipentosidine raised in rabbit (5 μl/ml) [28], and purified anti-CML (AGE) polyclonal antibody (5 μl/ml) [29] or isotype control antibody (Vector Laboratories Inc., Burlingame, CA, USA). The major epitope structure of anti-AGE antibody is identified as N^{ϵ}-carboxymethyllysine (CML) [29]. Biotin-labeled goat antirabbit IgG serum (diluted 1:200, Vector Laboratories Inc.) was used as the secondary antibody. All the glass slides were then incubated with avidin-biotininylated horseradish peroxidase macromolecular complex (ABC Kit, Vectastain, Vector Laboratories Inc.), followed by the peroxidase substrate 3,3'-diaminobenzidine (DAB, Vector Laboratories Inc.). Absorption tests with antibody preincubated with nitrotyrosine-labeled protein prevented the immunostaining, demonstrating the specificity of the antibody.

Western blotting for iNOS protein

After feeding piroxicam for 2 weeks, mice were euthanized and the colon was removed, rinsed in ice-cold PBS, and homogenized in lysis buffer (100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, in PBS pH 7.2). After centrifuging at 15,000 rpm for 20 min at 4°C, the supernatant was used for the assay. Protein concentration was measured using a commercial reagent based on BCA staining from Pierce Biotechnology, Inc. Equal amounts (25 μg) of protein were loaded onto a 7.5% polyacrylamide gel and separated by electrophoresis (100 V, 90 min). Proteins were then transferred to nitrocellulose (0.14 A, 12 h) and the membrane was blocked with 5% nonfat dry milk. The membrane was incubated with a rabbit polyclonal primary anti-iNOS antibody (1:5000, BD Transduction, San Diego, CA, USA) overnight at 4°C. Antibody labeling was detected using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Newark, NJ, USA), according to the manufacturer's instructions. Specificity of the antibody was confirmed with the use of mouse macrophage RAW 264.7 cell lysate stimulated with IFN-γ and LPS (BD Transduction). Equivalent loading of protein was verified by Coomassie blue staining of the membrane. Films of Western blots were scanned in at 600 dpi using an Epson Expression 1600 scanner (Epson America, Long Beach, CA, USA).

Immunohistochemical detection of 8-hydroxy-2'-deoxyguanosine

Purified mouse monoclonal antibody against 8-hydroxy-2″-deoxyguanosine N45.1 (10 μg/ml) was used for the immunostaining of 8-hydroxy-2'-deoxyguanosine (8-OHdG) [30]. Colon specimens were fixed in neutral-buffered formalin solution and embedded in paraffin. The antigen retrieval was performed by autoclaving the slides at 121°C for 5 min in 10 mM citrate buffer. The avidin-biotin complex method with alkaline phosphatase (Vector Laboratories Inc.) was used for the immunohistochemical study.

Treatment of mice with diphenylene iodiniumchloride (DPI)

IL10-deficient mice were placed on piroxicam-containing diet as described above. With initiation of NSAID treatment, mice were randomized into two groups. DPI was dissolved in a minimal volume of dimethylsulfoxide (DMSO) and diluted with sterile phosphate-buffered saline (PBS). Mice received 20 μmol/kg of DPI by intraperitoneal injection [31]. Control mice received injections of solvent (DMSO in PBS). Injections were repeated daily. Severity of colitis was determined by analysis of histopathology at day 7 or day 14 after the initiation of treatment.

Analysis of histopatholgy

Samples from the entire gastrointestinal tract were examined by the same pathologist (R.G.L.) without knowledge of which treatment group the samples were from. Because intestinal lesions were multifocal and of variable severity, the grades given to any section of intestine took into account the number of lesions as well as their severity. A grade from 0 to 4 was based on the following criteria:

1) Grade 0 indicates no change from normal tissue.
2) Grade 1 indicates one or a few multifocal mononuclear cell infiltrates in the lamina propria, accompanied by minimal epithelial hyperplasia and slight to no depletion of mucus from goblet cells.
3) Grade 2 indicates lesions were more frequent and typical changes included several multifocal, mild inflammatory cell infiltrates in the lamina propria, composed primarily of mononuclear cells with a few neutrophils. Mild epithelial hyperplasia and mucin depletion were also seen. Small epithelial erosions were occasionally present and inflammation rarely involved the submucosa.
4) Grade 3 indicates lesions involved a large area of the mucosa or were more frequent than grade 2 lesions. Inflammation was moderate and often involved the submucosa but was rarely transmural. Inflammatory cells were a mixture of mononuclear cells as well as neutrophils, and crypt abscesses were sometimes observed. Moderate epithelial hyperplasia and mucin

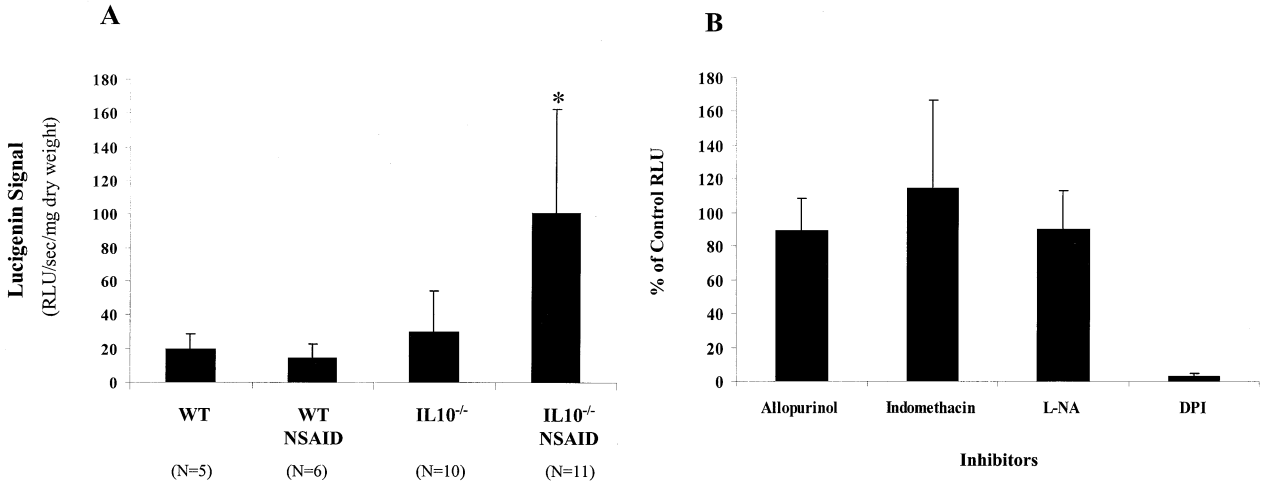

Fig. 1. (A) The production of lucigenin-amplified chemiluminescence in wild-type (wt) and IL10$^{-/-}$ colon. Chemiluminescence from inflamed colonic tissue of IL10$^{-/-}$ mice treated with 200 ppm piroxicam diet was significantly raised compared with colonic tissues from wt mice and IL10$^{-/-}$ mice with control diet. Values are expressed as mean ± SD. Data are representative of three independent experiments. *$p < .01$ compared with wt control, wt treated with piroxicam, and IL10$^{-/-}$ control. (B) The effect of pharmacological agents on chemiluminescence of colonic strips. Colonic tissue strips were preincubated with allopurinol (100 μM), indomethacin (100 μM), L-NA (100 μM), or DPI (100 μM), and chemiluminscence was measured in each case. The data are presented as mean percentages of chemiluminescence values of control inflamed colonic strip from five NSAID-treated IL10$^{-/-}$ mice.

depletion were seen. Ulcers were occasionally observed.

5) Grade 4 indicates lesions usually involved most of the intestinal section and were more severe than grade 3 lesions. Inflammation was intense, including mononuclear cells and neutrophils, and was sometimes transmural. Epithelial hyperplasia was marked with crowding of epithelial cells in elongated glands. Few mucin-containing cells were seen. Crypt abscesses and ulcers were present and foci of fibrinoid necrosis were present in the submucosa contiguous to ulcerations and crypt abscesses.

Statistical analysis

The data are expressed as means ± SD. Differences among the groups were tested using one-way analysis of variance (ANOVA) followed by Scheffe's test. The level of significance was $p < .05$.

All data handling and statistics generating were performed using the statistical software package StatView 5.0 for Windows (SAS Institute Inc., Cary, NC, USA).

RESULTS

Lucigenin-amplified chemiluminescence of colon tissue from wt and IL10$^{-/-}$ mice

Superoxide production in the colonic tissue was estimated by measuring lucigenin-enhanced chemiluminescence. Mean chemiluminescence of wild-type (wt) colon was not changed with piroxicam treatment (19.9 ± 8.9 vs. 14.4 ± 8.1 RLU/s/mg tissue, Fig. 1A). The mean chemiluminescence values of IL10$^{-/-}$ control mice were not statistically significantly higher that those of wt mice (30.1 ± 23.9). The mean chemiluminescence of colons from NSAID-treated IL10$^{-/-}$ mice was 101.2 ± 61, more than 5-fold higher than those of wt mice (control and NSAID-treated) and greater than 3-fold higher than control IL10$^{-/-}$ colons ($p < .01$, Fig. 1A).

To assess the source of the reactive oxygen species, colonic tissue was incubated in the presence of the following inhibitors: (i) DPI, an inhibitor of flavin-containing enzymes; (ii) allopurinol, an inhibitor of xanthine oxidase; (iii) L-NA, a nonspecific inhibitor of nitric oxide synthase enzymes; and, (iv) indomethacin, a nonselective cyclooxygenase inhibitor. Nearly 100% inhibition of chemiluminescence was obtained with the use of DPI, indicating that flavin-containing enzymes such as NADPH oxidase were the source of the ROS (Fig. 1B). No significant inhibition was seen with the other inhibitors. To determine if superoxide or hydrogen peroxide were important ROS in this mechanism, colonic tissue was incubated in the presence of superoxide dismutase or catalase. Addition of superoxide dismutase to the buffer resulted in a 40% inhibition of lucigenin-enhanced chemiluminescence, demonstrating a contribution of superoxide. In contrast, no inhibition of the chemiluminescence was seen with the addition of catalase, indicating that peroxide production did not contribute to the lucigenin-enhanced chemiluminescence (data not shown).

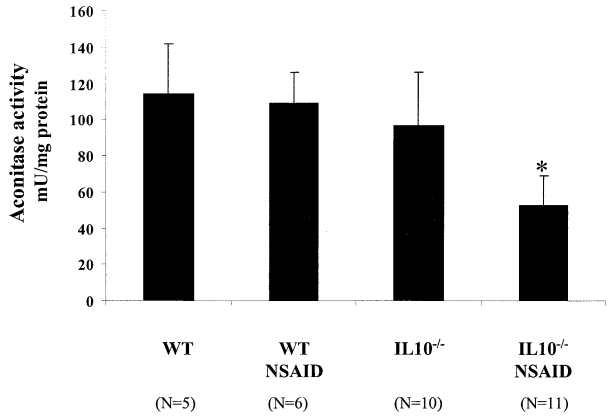

Fig. 2. Aconitase activity in the colon of wt and IL10$^{-/-}$ mice. Aconitase activity in colonic tissue was determined by measurement of the decrease in *cis*-aconitate over time. In IL10$^{-/-}$ mice treated with piroxicam, the aconitase activity in the colonic tissues was reduced to 50% compared to those in the other three groups in the direct assay. Values are expressed as mean ± SD. Data are representative of two independent experiments. *$p < .01$ compared with wt control, wt treated with piroxicam, and IL10$^{-/-}$ control.

Aconitase activity in the colon of wt and IL10$^{-/-}$ mice

To evaluate for evidence of oxidative stress, aconitase activity in the colonic tissues from wt and IL10$^{-/-}$ mice was measured. Aconitase is inactivated by superoxide radical [32,33]. Two independent assay methods were used for the same samples. In the direct assay, we measured the decrease in the enzyme substrate cis-aconitate in the buffer. In the coupled assay, citrate is metabolized to aconitate with concomitant formation of NADPH from $NADP^+$ by isocitrate dehydrogenase. Activity is determined by the measurement of NADPH production.

There were no significant differences in colonic aconitase activity among the three groups—wt control, wt treated with piroxicam, and IL10$^{-/-}$ control—of mice. In addition, after 1 week of NSAID treatment, there was no significant difference in colonic aconitase activity between NSAID-treated and control IL10$^{-/-}$ mice (data not shown). However, after 2 weeks of NSAID treatment of IL10$^{-/-}$ mice, the colonic aconitase activity was decreased 50% as compared to control IL10$^{-/-}$ and wt mice, when measured using both of these assay methods (Fig. 2). Although the aconitase activity was higher in the direct method, as reported by Kennedy et al. [23], the relative activity vs. wt control did not differ in either method (data not shown).

Glutathione levels in colonic mucosa

The content of GSH, GSSG, and related parameters in the colonic mucosa are shown in Table 1. Total glutathione levels in colonic mucosa did not differ among wt, IL10$^{-/-}$ control, and IL10$^{-/-}$ treated with piroxicam mice. However, GSSG, an oxidized product of glutathione, increased approximately 2-fold in the colons of piroxicam-treated colitis IL10$^{-/-}$ mice as compared with wt control, wt treated with piroxicam, and IL10$^{-/-}$ control groups ($p < .01$, Table 1). There was no significant difference in the glutathione content in the colonic mucosa between wt control and wt treated with 200 ppm piroxicam for 2 weeks (data not shown).

Evidence for advanced glycation endproducts in the colon of NSAID-treated IL10$^{-/-}$ mice

Immunohistochemical studies for the advanced glycation endproducts CML and pentosidine were performed. In both control and piroxicam-treated wt mouse colon there was essentially no CML detected (data not shown). Faint immunostaining for CML was observed in the lamina propria and submucosa of the colonic tissues from control IL10$^{-/-}$ mice (Fig. 3A). In contrast, intense immunostaining for CML was detected in colonic tissue from NSAID-treated IL10$^{-/-}$ mice (Fig. 3B). Immunostaining with anti-CML was occasionally noted in epithelial cells at the top of the gland. Moreover, prominent staining was observed in the infiltrating inflammatory cells in the lamina propria and submucosa. The muscle layer also showed diffuse staining in mice with colitis (Fig. 3B). The antipentosidine antibody revealed minimal staining of colonic tissues from control wt (data not shown) or control IL10$^{-/-}$ mice (Fig. 3C). In contrast, diffuse staining was seen in areas of the lamina propria in NSAID-treated IL10$^{-/-}$ mice with severe inflammation (Fig. 3D). There was no qualitative difference in the staining pattern for the advanced glycation endproducts

Table 1. Glutathione Levels in Colonic Mucosa

Genotype	Number of mice	Diet	Total glutathione (nmol/mg protein)	$GSSG^{GSH\ eq}$ (nmol/mg protein)	$GSH^{moles}/GSSG^{moles}$
WT	6	Control	24.98 ± 3.92	0.32 ± 0.15	180.1 ± 73.2
IL10$^{-/-}$	7	Control	25.29 ± 5.03	0.38 ± 0.12	140.6 ± 58.3
IL10$^{-/-}$	7	Piroxicam	26.95 ± 4.93	0.86 ± 0.38*,†	70.3 ± 28.0*,†

Data are representative of two independent experiments.
* Significantly different from WT control ($p < .01$).
† Significantly different from IL10$^{-/-}$ control ($p < .01$).

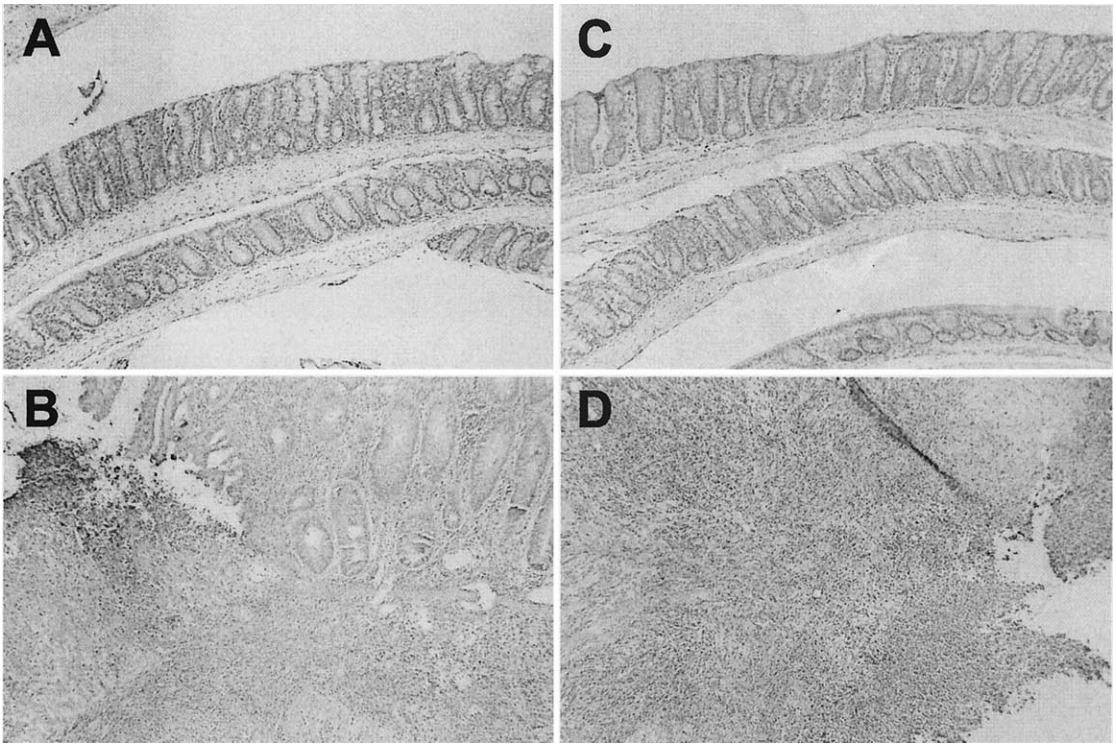

Fig. 3. Advanced glycation endproducts in control and NSAID-treated IL10$^{-/-}$ mice. Purified anti-*N*-carboxymethyl-lysine (CML) and antipentosidine polyclonal antibodies were used to reveal the distribution of AGEs in the colon. (A) Rare immunostaining for CML was observed in the lamina propria and submucosa of the colonic tissues in IL10$^{-/-}$ control mice (20×). (B) Prominent immunostaining for CML was observed in the lamina propria of NSAID-treated IL10$^{-/-}$ mice (20×). (C) Minimal immunostaining for pentosidine was observed in the lamina propria and submucosa of the colonic tissues in IL10$^{-/-}$ control mice (20×). (D) Prominent immunostaining for pentosidine was observed in the lamina propria of NSAID-treated IL10$^{-/-}$ mice (20×). Immunohistochemical staining with anti-CML and antipentosidine in colons of NSAID-treated IL10$^{-/-}$ mice was most prominent in the infiltrating inflammatory cells with only infrequent staining of overlying epithelium.

CML and pentosidine when assessed after 2 weeks of NSAID treatment, as compared to 4–6 weeks after the NSAID treatment was discontinued (data not shown).

Evidence for reactive nitrogen species

Having demonstrated increased nitric oxide (NO) production in IL10$^{-/-}$ mice previously [9,34], we assessed the expression of inducible NO synthase (iNOS) in NSAID-induced colitis. Western blot analysis for iNOS protein did not demonstrate expression in control and NSAID-treated wt mice. In contrast, low levels of iNOS expression were observed in control IL10$^{-/-}$ mice. Colons from NSAID-treated IL10$^{-/-}$ mice had a significant increase in iNOS expression (Fig. 4A).

Next, colonic tissues were examined by immunohistochemistry for the presence of nitrotyrosine, a marker of NO$^{\bullet}$-induced damage to proteins. Tyrosine residues are nitrosylated in the presence of peroxynitrite and other reactive nitrogen species. Thus, elevated levels of nitrotyrosine are consistent with increased levels of reactive oxygen and nitrogen species. Rare epithelial cells of control wt mice and IL10$^{-/-}$ mice had mild immunostaining with antinitrotyrosine antibody. Rare cells in the lamina propria also showed positive staining with the antibody (Fig. 4B). However, in colonic tissue from NSAID-treated IL10$^{-/-}$ mice, epithelial cells were strongly stained with antinitrotyrosine antibody and abundant infiltrate inflammatory cells both in lamina propria and submucosa also were strongly positive (Fig. 4C).

8-OHdG immunostaining

To assess for evidence of oxidative DNA damage in the colons of IL10$^{-/-}$ mice colitis, we performed immunohistochemical analysis using a purified monoclonal antibody directed against 8-OHdG (N45.1). The staining of 8-OHdG was predominantly confined to nuclei of the colonic tissue. Nuclei of normal epithelial cells from wt control and control young IL10$^{-/-}$ mice showed faint immunostaining. Epithelial cells in the colon of wt mice fed piroxicam showed weak staining of nuclei (data not shown). Colonic epithelium of IL10$^{-/-}$ mice treated with piroxicam for 2 weeks demonstrated moderate immunostaining, and the staining became much stronger in

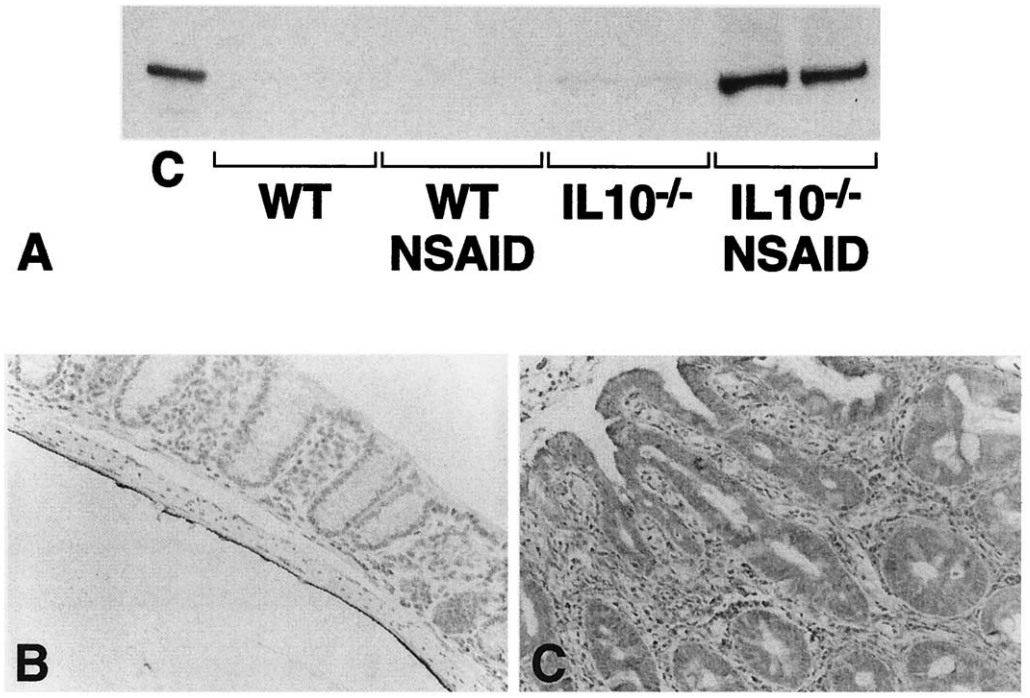

Fig. 4. (A) Western blot analysis for inducible nitric oxide synthase expression. Equivalent amounts of protein (25 μg) isolated from control and piroxicam-treated wt and IL10$^{-/-}$ mouse colons were separated on 7.5% SDS-PAGE, and iNOS expression was assessed by Western blot analysis. C = control, lysate of LPS-stimulated RAW 264.7 mouse macrophage cell line. iNOS was not detected in either control (lanes 2 and 3) or piroxicam-treated wt mice (lanes 4 and 5). Low levels of expression were noted in control IL10$^{-/-}$ mice (lanes 6 and 7). In piroxicam-treated IL10$^{-/-}$ mice (lanes 8 and 9), iNOS expression was markedly increased as compared with control IL10$^{-/-}$ mice. (B) Immunohistochemical staining for nitrotyrosine in the colonic tissues from IL10$^{-/-}$ mice. In the control IL10$^{-/-}$, rare epithelial cells of the colon were lightly stained with antinitrotyrosine antibody. Only a few lymphocytes in the lamina propria showed positive staining with the antibody. (C) NSAID-treated IL10$^{-/-}$ mouse colon. Epithelial cells demonstrated strong staining with antinitrotyrosine. There is also a significant increase in the numbers of positively stained infiltrating inflammatory cells in the lamina propria.

the nuclei of colonic epithelial cells in IL10$^{-/-}$ mice with chronic colitis. Strong immunostaining was also observed in the infiltrating mononuclear cells of chronic colitis (Fig. 5).

DPI treatment of NSAID-treated IL10$^{-/-}$ mice

As our studies indicated the presence of oxidative stress in NSAID-treated IL10$^{-/-}$ mice, we simultaneously treated IL10$^{-/-}$ mice with piroxicam and DPI, an inhibitor of the flavoprotein-mediated reaction of both NADPH oxidase and NO synthase, and subsequently assessed the effect of DPI treatment on intestinal histopathology. DPI treatment of control IL10$^{-/-}$ mice had no effect on the normal colonic histology (data not shown). In contrast, NSAID-treated IL10$^{-/-}$ mice that also received DPI had a nearly 50% reduction in histopathologic score at both day 7 and day 14 of treatment (Fig. 6).

DISCUSSION

We have used NSAID-treated IL10-deficient mice to determine the extent to which development of IBD in this animal model of human disease is accompanied by oxidative stress. Our studies show that colonic tissue from NSAID-treated IL10$^{-/-}$ mice has a markedly increased level of ROS from flavin-containing enzymes. We also found that colonic tissue from NSAID-treated IL10$^{-/-}$ mice has increased levels of the oxidized form of glutathione (GSSG), as well as positive immunostaining for advanced glycation endproducts and 8-OHdG, indicating the presence of oxidative damage. Moreover, colons from NSAID-treated IL10$^{-/-}$ mice have increased expression of iNOS and increased levels of nitrotyrosine. Taken together, these data indicate that NSAID-induced IBD in IL10$^{-/-}$ mice is accompanied by increased ROS, RNS, and oxidative stress.

IL10 is known to deactivate macrophages and block the induced synthesis of inflammatory cytokines such as TNF-α and IL1 [6,35], which are known to induce the production of ROS and RNS. IL10 is also reported to directly inhibit free radical generation and IκB degradation in a mouse macrophage cell line [36]. Previously, we have found that IL10$^{-/-}$ mice develop a spontaneous inflammatory bowel disease that is very similar to human

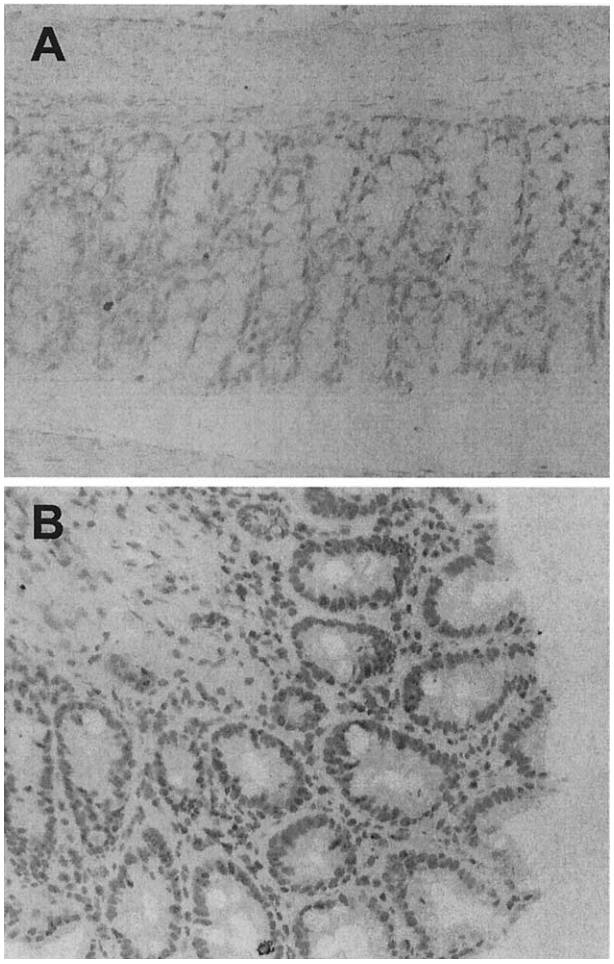

Fig. 5. Immunohistochemical detection of 8-OHdG in colonic epithelial cells of IL10$^{-/-}$ mice. (A) Nuclei of the normal epithelial cells from control young IL10$^{-/-}$ mice showed faint immunostaining. Rare lamina propria cells demonstrated strong staining. (B) Nuclei of colonic epithelium in NSAID-treated IL10$^{-/-}$ mice with chronic colitis showed strong staining with N45.1. Strong immunostaining was also obtained in the infiltrating cells in all of the specimens.

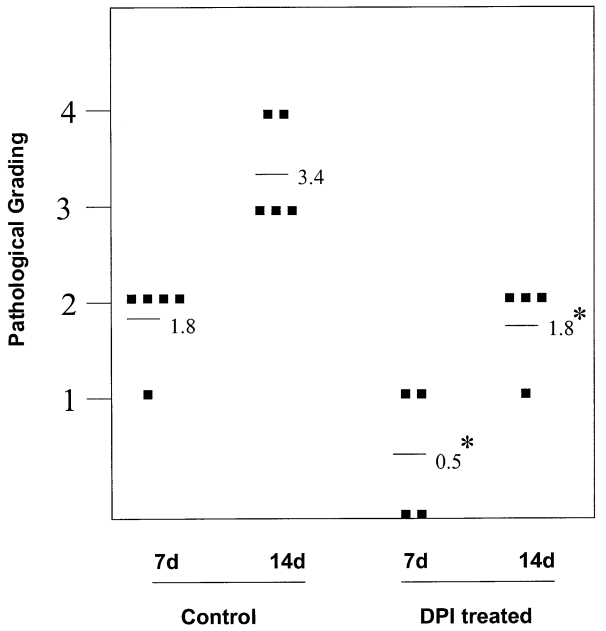

Fig. 6. The effect of DPI treatment on NSAID-induced colitis in IL10$^{-/-}$ mice. DPI treatment (20 μmol/kg, ip, daily) was initiated simultaneously with feeding of piroxicam. Control NSAID-treated mice received daily injections of solvent. Histopathological evaluation was performed 7 and 14 d after the initiation of piroxicam treatment. Control IL10$^{-/-}$ mice treated with DPI alone had no evidence of colitis (pathological score of 0). $^{+}p < .05$ as compared to control IL10$^{-/-}$ mice.

IBD. Recently, we reported that NSAID-treated IL10$^{-/-}$ mice rapidly develop severe colitis within 2 weeks [13]. In the inflamed colon of NSAID-treated IL10$^{-/-}$ mice, there was a marked infiltration of macrophages and CD4^{+} T cells, as well as increased expression of inflammatory cytokine mRNA and IFN-γ production from lamina propria T cells. Since these inflammatory cytokines are reported to be potent inducers of NO and ROS, we assessed this model for evidence of oxidative stress

Chemiluminescence is a commonly used means to evaluate ROS generation and lucigenin is widely used as a probe for $O_2^{\bullet -}$ measurement [37–39]. Lucigenin-amplified chemiluminescence in the inflamed colonic tissues in IL10$^{-/-}$ mice was significantly higher than that of wt mice and IL10$^{-/-}$ control mice. This result strongly suggests that colons with colitis from IL10$^{-/-}$ mice have high levels of ROS, with a significant component of superoxide. Although we can not determine what type of cells are producing superoxide in the tissue, our result suggests that NADPH oxidase may play an important role in superoxide generation because the only inhibitor to significantly reduce the chemiluminescence signal was a flavoprotein inhibitor, diphenyleneiodonium chloride (DPI). In the inflamed colon of IL10$^{-/-}$ mice, we observed extensive infiltration of CD4+ cells in the lamina propria and F4/80+ macrophages in the lamina propria and submucosa [13]. We hypothesize that these infiltrating cells are the origin of the superoxide production in the colon. Recently, it has been suggested that the pg91^{phox} homologue is highly expressed in the human and rat colonic epithelial cells [40,41]. Although the function of these enzymes is not yet clear, the presence of this enzyme in the inflamed colonic epithelium suggests that it might have a role in the regulation of tissue redox status in response to intestinal bacteria. The contribution of epithelial phox homologues in this intestinal inflammatory state is not yet known.

We suspected that colonic mucosa from inflamed colons would have an altered redox status because we observed that colonic tissues from IL10$^{-/-}$ mice with IBD had increased levels of superoxide. Glutathione in the cells provides reducing equivalents for the reduction

of ROS and is oxidized to GSSG. Decreases in GSH levels and GSH/GSSG ratio in tissue is a marker for the effects of oxidative stress on the cellular redox status. Reduced glutathione has been reported to be a key antioxidant in the gastrointestinal mucosa and in an animal model of IBD. The increased GSSG in the colitis colons from $IL10^{-/-}$ mice suggests increased oxidative stress in the epithelial mucosa in the colons and an altered redox homeostasis in the inflamed mucosa. Homozygous mice that lack the genes of both *Gpx1* (glutathione peroxidase) and *Gpx2* (epithelium specific glutathione peroxidase) develop colitis consistent with IBD [42]. Glutathione peroxidases contribute nearly all of the GSH-dependent, H_2O_2-reducing activity in the distal gastrointestinal epithelium, suggesting that these enzymes, and sufficient levels of intracellular GSH, are essential for protecting the cells from oxidative stress in the colon.

Nieto et al. and others have shown a decrease of total GSH in the rat intestine with induced colitis by trinitrobenzenesulfonic acid (TNBS) [43–45], and studies using this model have demonstrated amelioration of colitis with GSH supplementation [46]. Interestingly, TNBS is able to react directly with GSH [46], which may explain the rapid depletion of GSH in this IBD model. In contrast, in individuals with IBD, GSH of colonic mucosa from control and active/inactive ulcerative colitis patients did not show any significant differences among the groups, while the levels of GSSG in the mucosa were increased in the active ulcerative colitis [47]. Similarly, in our study, we could not observe a significant decrease of total glutathione in the inflamed colonic mucosa from $IL10^{-/-}$ mice 2 weeks after feeding them piroxicam. Although an increase in the expression of γ-glutamylcysteine synthetase (the enzyme catalyzing the rate-limiting step in GSH synthesis) has been reported in the rat model of TNBS-induced colitis [46], we did not detect by Western blot analysis an increase in the expression of this enzyme in this model of IBD (data not shown).

To further assess for evidence of oxidative damage, we measured cellular aconitase activity in the colons from $IL10^{-/-}$ mice treated with piroxicam. Aconitase, found in both the mitochondria and the cytosol, is a target of oxidative stress because of the loss of Fe from the [4Fe-4S] cluster. This enzyme is thought to be a sensitive indicator of intracellular steady state $O_2^{\bullet -}$ levels [48–50]. A decrease in aconitase activity suggests an increase in oxidative stress. Aconitase activity in the colonic tissues was measured by two individual methods for the same samples; in both methods, after 2 weeks of NSAID treatment, the aconitase activity was reduced by half in the colonic tissues from $IL10^{-/-}$ with colitis mice as compared with the activity in control $IL10^{-/-}$ mice. Interestingly, after 1 week of NSAID treatment, the level of aconitase activity was not changed from the control, suggesting that the development of oxidative stress was a consequence of the inflammation. Although we did not investigate the aconitase activity in cytosolic or mitochondria individually, the result in our study strongly suggests that the colonic tissues had intracellular oxidative damage during the development of colitis in the $IL10^{-/-}$ mice.

Immunohistochemical approaches are widely used because they provide information on specific localization of the targeted molecules in the tissue. In this study, we used immunostaining with antibodies against specific markers of oxidative damage in the inflamed colonic specimens. Advanced glycation endproducts (AGEs) are formed during Maillard reaction by the combined process of nonenzymatic glycation and oxidation (glycoxidation). These endproducts have been implicated in the pathogenesis of several diseases including atherosclerosis and diabetic and uremic complications [29,51,52]. Anderson et al. suggested that the in vivo production of glycoaldehyde and other reactive aldehydes by a myeloperoxidase-catalyzed system of human phagocytes may play an important role in generating AGEs and damaging oxidatively tissues at the site of inflammation [53]. Macrophages at the site of inflammation in atherosclerosis stain positively for CML, suggesting increased AGE formation [54]. In NSAID-treated $IL10^{-/-}$ mice, immunostaining for AGEs was strong. Infiltrating inflammatory cells stained more intensely than epithelial cells with the antibodies for AGEs. Weak staining with antibodies for AGEs may underestimate oxidative stress to cells/tissues with a high turnover, such as intestinal epithelium, because AGEs are more likely to accumulate in tissues with slow metabolic turnover, such as collagen in the tissues and lens [55].

In addition to the oxidative damage by reactive oxygen species, reactive nitrogen species may play an important role in inflammation in the colon. NO is thought to promote inflammation by mediating chemotaxis of neutrophils and monocytes [56]. Moreover, NO reacts easily with superoxide anion radical to generate the peroxynitrite anion, which is a potent oxidant and thought to be more toxic. IL10 has the potential to regulate nitric oxide production both in vitro and in vivo [57,58]. Using $IL10^{-/-}$ mice, we found that the absence of IL10 results in the induction of iNOS mRNA in the spleen cells by LPS stimulation [59], elevation of NO in the serum [60], and elevated production of NO in the colon organ culture [9]. In this study, we demonstrated both increased iNOS expression and the presence of nitrotyrosine in the inflamed tissue. Tyrosine nitration is caused by peroxynitrite and the reaction of myeloperoxidase-generated hydrogen peroxide and nitrate that can be found in inflammatory conditions. Singer et al. showed that iNOS

protein is expressed in polymorphonuclear cells in the lumen and in limited numbers of mononuclear cells in the lamina propria in other than epithelial cells of the colon specimens from IBD patients [3].

However, the role of nitric oxide in the development of colitis is uncertain. Tissue damage was significantly diminished in iNOS knockout mice when colitis was induced by dextran sodium sulfate treatment [61]. On the other hand, inhibition of nitric oxide synthesis by aminoguanidine increased intestinal damage in the acute phase of rat colitis induced by TNBS [62]. Moreover, McCafferty et al. reported that the absence of iNOS in IL10/iNOS double knockout mice did not have any effect on the spontaneous development of colonic inflammation [63]. Further study will be required to determine the functional significance of RNS in the development of NSAID-induced colitis in IL10$^{-/-}$ mice. In this study, however, increased RNS are consistent with our other data and lend additional support to the hypothesis that oxidative stress is elevated in this model of IBD.

The DNA base-modified product 8-hydroxy-2'-deoxyguanidine is one of the most commonly used markers for the evaluation of oxidative damage of DNA. 8-OHdG is induced by hydroxy radical, singlet oxygen, or photodynamic action [30]. We clearly showed that oxidative stress in the nucleus is induced in the epithelial cells in the colitis colon. We also observed that the anti-8-OHdG staining was variable in the colons from IL10$^{-/-}$ mice treated with piroxicam for 2 weeks, and stronger immunostaining was observed in the colons from IL10$^{-/-}$ with chronic colitis. Previously, we reported that a high incidence of colorectal adenocarcinomas (67%) was observed in spontaneous colitis in IL10$^{-/-}$ mice [9]. In our NSAID-induced colitis model, when mice were placed on standard diet for several weeks after 2 weeks of piroxicam feeding, chronic inflammation with subsequent cellular atypia, nuclear and cytologic pleomorphism, and invasive epithelium were seen in the colon [13], suggesting the potential to progress to colon cancer. In our present study, we observed strong immunostaining with anti-8-OHdG in the nuclei of the colonic epithelium of chronic colitis in IL10$^{-/-}$ mice. Accumulation of DNA damage induced by oxidative stress in the epithelial cells in the colon could lead to the transformation of cells, increasing the risk of development of colon cancer. Moreover, increased oxidative stress may promote epithelial proliferation, as has been reported by Kondo et al. [64], for colorectal carcinoma, which may further predispose the epithelium to cellular transformation. Interestingly, we found intense 8-OHdG immunostaining in the infiltrating mononuclear cells in lamina propria and submucosa. This result was consistent with that of Kondo et al. [64], who observed strong immunostaining in the infiltrating mononuclear cells throughout the human colonic specimens, suggesting that the cells producing reactive oxygen species also may have evidence of oxidative damage.

Treatment with the flavoprotein inhibitor DPI resulted in a significant reduction in pathology in NSAID-treated IL10$^{-/-}$ mice. DPI can irreversibly inactivate NADPH oxidase and NO synthase [65,66]. Suppression of ROS/RNS from inflammatory cells may have led to decreased pathological damage as well as altered ROS/NO-dependent signal transduction pathways [67,68], potentially altering the immune response as well. However, DPI is not specific for NADPH oxidase or iNOS. DPI can inhibit other flavoproteins such as dehydrogenases, monooxygenases, and H_2O_2-producing oxidases [65]. Further studies will be required to determine the key source(s) of the ROS and the mechanism(s) by which their inhibition in turn inhibits the development of colitis in this model. Nevertheless, our findings do provide a link between oxidative stress and the development of IBD in this model.

IL10$^{-/-}$ mice serve as a well-established model of human inflammatory bowel disease and colitis-associated cancer. We have used a variation of this model, NSAID-treated IL10$^{-/-}$ mice, to address potential underlying mechanisms of the pathology of IBD. The goal of this study was to determine the extent to which IBD in NSAID-treated IL10$^{-/-}$ mice is accompanied by oxidative stress. We evaluated various markers for oxidative stress in the colons from wt and IL10$^{-/-}$ mice treated with piroxicam. The increased levels of ROS in the inflamed colon from IL10$^{-/-}$ mice directly suggest the increased risk of oxidative damage in these tissues. The change in the redox status of mucosal glutathione (increased GSSG) clearly indicates a cellular response to oxidative stress. Decreased aconitase activity and the detection of AGEs and nitrotyrosine in the colon from IL10$^{-/-}$ mice treated with piroxicam suggest the oxidative damage to protein by ROS and NO. Furthermore, increased immunostaining with anti-8-OHdG, a signature of oxidative damage to nucleic acids, is further evidence of oxidative stress in this model. Our finding that treatment with DPI significantly reduces pathology in NSAID-treated IL10$^{-/-}$ mice links oxidative stress with the development of pathology in this model. Taken together, the results from this study demonstrate significant oxidative stress in NSAID-induced IBD in IL10$^{-/-}$ mice. Increased oxidative stress may play an important role in the immunopathologic damage in IBD and may serve as a promoter of the development of inflammation-associated colon cancer.

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ABBREVIATIONS

AGE—advanced glycation endproduct
CML—carboxymethyllysine
COX—cyclooxygenase
DAB—3,3'-diaminobenzidine
DPI—diphenyleneiodinium chloride
8-OHdG—8-hydroxy-2'-deoxyguanosine
GSH—glutathione
GSSG—glutathione disulfide
IBD—inflammatory bowel disease
IFN-γ—interferon-γ
L-NA—N-ω-nitro-L-arginine
NADPH—nicotineamide adenine dinucleotide phosphate (reduced)
NSAID—nonsteroidal anti-inflammatory drug
PG—prostaglandin
TNBS—trinitrobenzenesulfonic acid
TNF-α—tumor necrosis factor α