Luminal Antioxidants Enhance the Effects of Mesalamine in the Treatment of Chemically Induced Colitis in Rats

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Previous experiments in rats with chemically induced colitis have shown that the antioxidant N-acetylcysteine plus mesalamine (5-ASA) exerted a significantly greater therapeutic effect in promoting mucosal healing when compared to either agent alone. The aims of the present study were to compare the effects of three antioxidants plus mesalamine vs. 5-ASA alone in treatment of colitis induced by trinitrobenzene sulfonic acid (TNBS) in rats. *Methods:* Three days following induction of TNBS colitis, rats received 8 days of rectal therapy with 5-ASA, or 5-ASA plus vitamin C (ascorbic acid), 5-ASA plus phenyl butylnitrone (PBN) and 5-ASA plus vitamin E (α-tocopherol). Distal colonic tissues were examined for microscopic colitis and myeloperoxidase (MPO) activity. *Results:* Global assessments of microscopic colitis induced by TNBS indicated that 5-ASA alone significantly changed colonic injury by −31%. Combination therapy with ascorbic acid plus 5-ASA or α-tocopherol plus 5-ASA caused further significant change in TNBS colitis by −65 and −82%, respectively. Each of these values was significantly below scores observed with 5-ASA as monotherapy. Reduction in colitis with PBN plus 5-ASA was not different from 5-ASA alone. MPO activity was decreased significantly in response to monotherapy with 5-ASA and each of the antioxidants plus 5-ASA when compared to TNBS. α-Tocopherol plus 5-ASA, however, was the only treatment strategy that reduced significantly MPO activity below that recorded for 5-ASA alone. In conclusion, our results indicate that antioxidants other than N-acetylcysteine significantly enhance the therapeutic effectiveness of 5-ASA in the treatment of TNBS colitis. α-Tocopherol plus 5-ASA exerted profound anti-inflammatory and reparative effects upon colitis induced by TNBS. Exp Biol Med 233:1301–1308, 2008

Key words: ascorbic acid; α-tocopherol; phenyl butylnitrone; 5-ASA; colitis

Introduction

Reactive oxygen species (ROS) have been associated with the initiation or aggravation of a number of illnesses including inflammatory bowel disease (IBD) (1–3). Excessive generation of ROS can induce biochemical alterations in macromolecules, such as lipids, proteins, and DNA, thereby inducing cellular injury or death in the inflammatory process. Alternatively, the duality of ROS effects is reflected by the ability of these oxygen and nitrogen radicals to positively affect intracellular signaling pathways and metabolism (4–7). In this latter instance, ROS are formed as byproducts of oxygen metabolism under physiological or non-stress conditions and their levels are regulated by both non-enzymatic and enzymatic antioxidants (1, 8). Oxidative stress encountered in inflammatory states and in ischemia and reperfusion injury represents a disequilibrium or imbalance between the generation of reactive oxygen intermediates and the removal of these species by antioxidant systems. Exposure of cells to oxygen radicals can activate or trigger surface receptors and cellular pathways that can promote either cell survival or death. The fate of the cell experiencing oxidative stress depends, in part, on the cell type and the duration and magnitude of exposure and the dominance of opposing cellular signals (6–8).

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Therapeutic approaches to clinical and experimental forms of IBD have, heretofore, been broad based and nonselective with aims to suppress the inflammatory process and immune overreactivity. These strategies have reflected our incomplete and fragmented understanding of the pathogenesis of ulcerative colitis and Crohn's disease. More recent investigations have expanded and emphasized the apparent linkage between genetics, immune cell function and luminal bacteria recognition in these chronic inflammatory diseases (9). Mesalamine (5 aminosalicylic acid; 5-ASA) has been used in various oral and enema/suppository formulations for mild to moderate ulcerative colitis and also in Crohn's disease (10). 5-ASA exerts its anti-inflammatory effects through several pharmacological actions. These include its antioxidant properties and the ability to inhibit prostaglandin synthesis and reduce nuclear factor-kB (NF-kB) activation (11, 12). The role of oxidative stress in experimental models of colitis has been investigated directly and indirectly in studies employing topical and systemic antioxidant agents (13–19). Improvement in measures of inflammation by these agents has been ascribed to reduction in ROS, inhibition of nitric oxide synthase (NOS) and inhibition of the activation of NF-kB. These studies indicate the presence of similarities in certain aspects of the pharmacological actions of mesalamine and antioxidants.

Therapeutic interventions in experimental colitis in our laboratory have examined the ability of the antioxidant N-acetylcysteine (NAC) in combination with mesalamine to reduce inflammation and promote healing in trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats (20, 21). The rationale for this therapeutic approach was to take advantage of the shared and distinctive actions of each agent in a combined formulation. This pharmacological approach offered the prospect of being able to modulate a number of components of the inflammatory cascade in an immune-mediated model of colitis. Experimental results indicated that the combination of N-acetylcysteine plus 5-ASA when administered rectally to rats with TNBS colitis was superior to either agent alone in promoting mucosal healing and reducing inflammation (20). Furthermore, combination therapy resulted in significant reductions in colonic myeloperoxidase (MPO) activity and proinflammatory cytokine gene expression. Additional studies have demonstrated that NAC plus 5-ASA exerted its therapeutic benefit, in part, by inhibiting the proinflammatory actions of prostaglandin E_2 (PGE_2) and offsetting the deleterious effects of oxidative and nitrosative stress induced by TNBS colitis (21).

The aims of the present study were to address the question of whether antioxidants other than N-acetylcysteine, in combination with 5-ASA, could affect the ulcerative and inflammatory responses to TNBS and promote mucosal repair. The antioxidant agents that were examined were vitamin C (ascorbic acid), vitamin E (α-tocopherol) and phenyl butylnitrone. Ascorbic acid is a water soluble vitamin and α-tocopherol is a lipid soluble vitamin. The antioxidant property of each substance relates to their ability to serve as electron donors and therefore reducing agents capable of interrupting free radical chain processes (22, 23). Phenyl butylnitrone is a member of a family of nitrones used as spin-trapping agents to trap or scavenge free radicals (24). The pharmacologic effects of PBN have been studied in a number of experimental diseases including dextran sulfate sodium-induced colitis in mice (19). Responses to combination therapies, delivered topically to the distal colon, were compared to 5-ASA alone.

Materials and Methods

Experimental Animals. Male Sprague Dawley rats weighing 200–250 grams were housed in the animal facility in cages containing contact bedding. Rats were deprived of food but not water for 24 hrs prior to the induction of colitis. Institutional approval for experimental protocols was provided by the research and animal care committees of the Research Services at the Oklahoma City Veterans Administration Medical Center.

Induction of Colitis. Fasted animals were lightly anesthetized with isoflurane and then subjected to enema administration of TNBS and subsequently study drugs. The experimental protocol is similar to that previously described (20). The tip of a polyethylene catheter was advanced transanally 8 cm to the distal colon and a single dose of TNBS (Sigma, St. Louis, MO), was instilled intraluminally (15 mg of TNBS dissolved in 0.6 ml volume of 50% ethanol) to induce colitis. Following the administration of TNBS the animals were maintained in a head-down position for approximately 60 seconds to prevent leakage of the infusate.

Three days after TNBS dosing rats were randomly assigned to receive one of the following treatments by enema in a manner similar to that used for TNBS administration. The TNBS treated animals received 5-ASA (100 mg/kg) alone or 5-ASA in combination with: ascorbic acid (100 mg/kg), phenyl butylnitrone (PBN; 30 mg/kg) and α-tocopherol (DL-all-rac-α-tocopherol, 50 mg/kg solubilized in DMSO). Antioxidants were purchased from Sigma, St. Louis, MO. The doses of selected antioxidants were derived from the literature in which these agents had been used in experimental models of disease or IBD (19, 22, 23). Comparator groups included TNBS dosed rats that received doses of rectal saline for 8 days and saline treated controls. Rats were treated for 8 days with a single daily dose of test agent(s) by enema. Animals were sacrificed 24 h after last enema treatment.

Assessment of Macroscopic Injury. Upon sacrifice, the distal 10 cm of the colon was removed, opened with a longitudinal incision, and rinsed with phosphate-buffered saline to remove fecal material. Macroscopic assessment of colitis was carried out by an independent observer who was unaware of the treatment groups. The criteria and scale of grading macroscopic injury are listed in Table 1. Inflam-

Table 1.

Macroscopic injury	Score
No damage	0
Hyperemia, no ulcers	1
Linear ulcer with no significant inflammation	2
Linear ulcer with inflammation at one site	3
Two or more sites of ulceration and inflammation	4
Two or more major sites of ulceration and inflammation or one site of ulceration/ inflammation up to 1 cm along the length of colon	5
Damage >1 and <2 cm along the length of the colon; the score is increased by 1 for each additional cm of involvement	6

Table 2.

Criteria for assessment of microscopic injury	
Epithelial damage	0 = Normal 1 = Focal mucosal injury 2 = Moderate mucosal injury 3 = Extensive mucosal injury
Architectural damage	0 = Normal 1 = Mildly disturbed 2 = Moderately disturbed 3 = Severely disturbed
Mononuclear infiltration	0 = Normal 1 = Mild increase 2 = Moderate increase 3 = Severe increase
PMN infiltration	0 = Normal 1 = Present in surface epithelium 2 = Cryptitis 3 = Crypt abscess
Ulceration	0 = None 1 = 1%–33% ulcerated 2 = 34%–66% ulcerated 3 = ≥67% ulcerated

mation was present if the mucosa was erythematous. Ulceration of the mucosa was defined as a distinct break or interruption of the mucosa. Once macroscopic damage was assessed full thickness colonic tissue samples were taken from inflamed areas and processed for histology.

Assessment of Microscopic Injury. Histological assessment was used to measure colonic injury. Colonic tissue samples taken from the distal colon were immersed in 10% phosphate buffered formalin and subsequently embedded in paraffin. Sections of 3 micron thickness were stained with hematoxylin and eosin. The slides were then evaluated by a pathologist for epithelial damage, architectural changes, polymorphonuclear leukocyte (PMN) infiltration, mononuclear cell infiltration, and ulceration. The microscopic features of colitis were graded on a 0–3 scale with 0 being normal and 3 representing severe or most intense abnormality. Table 2 depicts the criteria used for assessment of microscopic injury. In addition to scoring individual features of colitis, an aggregate score of colitis for each rat was tabulated by adding together individual parameter scores, thus providing a global assessment of colitis.

Assessment of Colonic Inflammation by Myeloperoxidase Assay. Myeloperoxidase activity was measured in colonic tissue samples by colorimetric assay. Whole-thickness tissues from the distal colon were weighed (100 mg) and immediately snap-frozen in liquid nitrogen for storage at –80°C. The tissues were then removed from storage and allowed to thaw on ice. Once thawed, 1 ml of hexadecyltrimethylammonium bromide (HTAB; Sigma, St. Louis, MO) containing 50 mM KH_2PO_4 (Sigma) and 0.1 M Na_2HPO_4 (Sigma) was added, homogenized and the resultant suspension was then centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was collected for measurement of MPO activity. Horseradish peroxidase (Sigma) was used as a standard; stock solution of 0.5 mg/ml. Tetramethylbenzidine (TMB; Sigma) was used as the substrate for carrying out the reaction. At the time of assay, 25 μl of standard and sample were added to appropriately labeled tubes. TMB was added at a volume of 250 μl to initiate the reaction and 0.1 M H_2SO_4 (250 μl) was added after 10 min to terminate the reaction. The absorbance changes were read at 450 nm and recorded. Results are expressed as nanograms per gram of tissue. MPO activity was used as an indirect measure of the severity of colonic inflammation reflected by PMN leukocyte infiltration.

Statistical Analysis. All values in the figures and text are expressed as means ± standard error of the mean (SEM). The statistical significance of any difference among groups was analyzed using Student's two-tailed *t* test for equal and unequal variance observations. *P* values of < 0.05 were considered to be statistically significant.

Results

Effects of Antioxidant Plus 5-ASA Therapy on Macroscopic Colitis. Visual evidence of colitis 11 days after TNBS administration was scored 5.9 ± 0.6 (Fig. 1). Monotherapy with 5-ASA alone resulted in significant reduction in TNBS induced macroscopic injury, 3.8 ± 0.4 ($P < 0.01$). Addition of ascorbic acid and α-tocopherol to 5-ASA resulted in significant reduction in TNBS-induced macroscopic injury to values of 3.8 ± 0.5 and 3.2 ± 0.5 ($P < 0.05$ and $P < 0.01$), respectively. These results, however, were not different from those achieved with 5-ASA alone. Combination of PBN with 5-ASA did not result in significant reduction in TNBS induced colitis.

Effects of Antioxidant Plus 5-ASA Therapy on Microscopic Colitis. Global assessments of microscopic colitis induced by TNBS (score 13.3 ± 0.5) indicated that 5-ASA alone significantly reduced colonic injury by 31% (9.1 ± 1.0; $P < 0.01$) (Fig. 2). Combination therapy with either ascorbic acid plus 5-ASA or α-tocopherol plus 5-ASA caused further significant reduction in TNBS colitis and resulted in injury scores of 4.7 ± 0.9 (–65%) and 2.4 ± 1.0

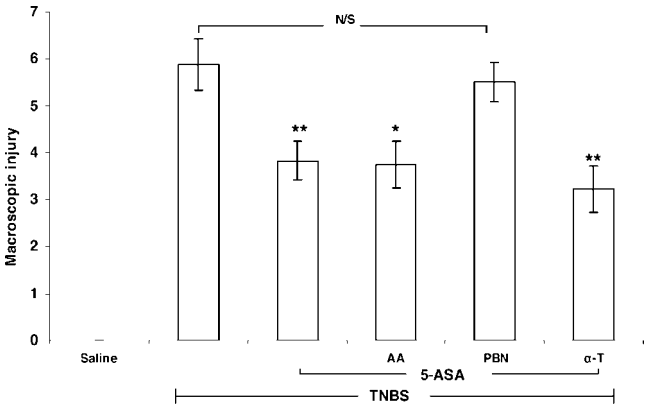

Figure 1. Macroscopic injury scores from the distal colon of animals 11 days after TNBS and 8 days of treatment. Animals treated with saline alone served as control group. Treatment protocols were initiated 3 days after induction of TNBS colitis and comprised daily rectal dosing with saline, 5-ASA (100 mg/kg), ascorbic acid (AA; 100 mg/kg) + 5-ASA, PBN (30 mg/kg) + 5-ASA and α-tocopherol (α-T, 50mg/kg) + 5-ASA. * $P < 0.05$ and ** $P < 0.01$ vs TNBS alone. N/S denotes not significant.

(–82%), respectively. Each of these values was not only significantly less than that observed with TNBS but they were also significantly below scores observed with 5-ASA as monotherapy ($P < 0.01$ and $P < 0.001$), respectively. Enema therapy with PBN plus 5-ASA resulted in a colitis injury score of 7.5 ± 1.4, which was significantly less than TNBS ($P < 0.01$) but was not different from results with 5-ASA alone (9.1 ± 1.0; $P > 0.1$).

Effects of Luminal Antioxidant Therapies Plus Mesalamine on Individual Measures of Microscopic Colitis. Subset analysis of the separate histological indices of colitis for each antioxidant plus 5-ASA was compared to responses to 5-ASA alone in the treatment of TNBS colitis (Fig. 3). Figure 3A indicates that combination therapy with ascorbic acid plus 5-ASA caused numerical reductions in 5

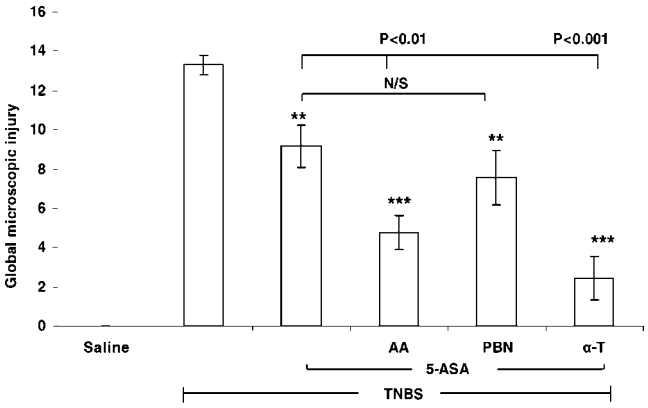

Figure 2. Global microscopic injury scores from distal colon of animals 11 days after TNBS and 8 days of treatment. Individual treatments were initiated 3 days after induction of TNBS colitis and included daily administration of one of the following: 5-ASA (100 mg/kg), ascorbic acid (AA; 100 mg/kg) + 5-ASA, PBN (30 mg/kg) + 5-ASA and α-tocopherol (α-T, 50mg/kg) + 5-ASA. ** $P < 0.01$, *** $P < 0.0001$ vs TNBS alone. N/S denotes not significant.

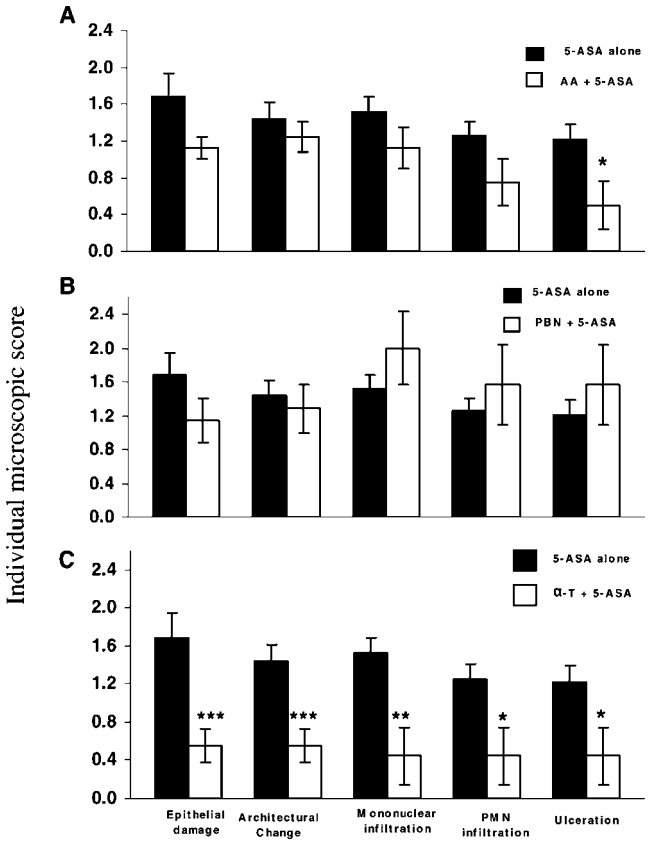

Figure 3. Subset analysis of individual indices of TNBS-induced microscopic colitis results with 5-ASA alone are compared to responses with each antioxidant plus 5-ASA. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs 5-ASA only.

of the 6 measures of colitis, but these values did not reach statistical significance. Mucosal ulceration, however, was significantly inhibited by ascorbic acid plus 5-ASA. The combination of PBN plus 5-ASA caused a variable response in the histological features of colitis and none of these was different from monotherapy with 5-ASA (Fig. 3B). In contrast, topical therapy with α-tocopherol plus 5-ASA caused significant decreases in each histological feature of colitis when compared to 5-ASA alone (Fig. 3C).

Representative photomicrographs of distal colonic specimens from rats subjected to the different treatment protocols are depicted in Figure 4. TNBS colitis, illustrated by the histological specimen obtained 11 days after induction of colitis (Fig. 4B), was characterized by mucosal ulceration, intense inflammatory cell response in the lamina propria, cystic glandular dilatation and the presence of inflammatory cells within the glandular lumen. In addition, there was a notable lack of goblet cells. Figure 4C shows the effects of monotherapy with topical 5-ASA, which was administered for 8 days and begun 3 days after initiation of TNBS colitis. This photomicrograph indicates an area of mucosal ulceration, mild inflammation in the submucosa, cystic glandular dilatation and restoration of goblet cells. Combination therapy with luminally delivered ascorbic acid

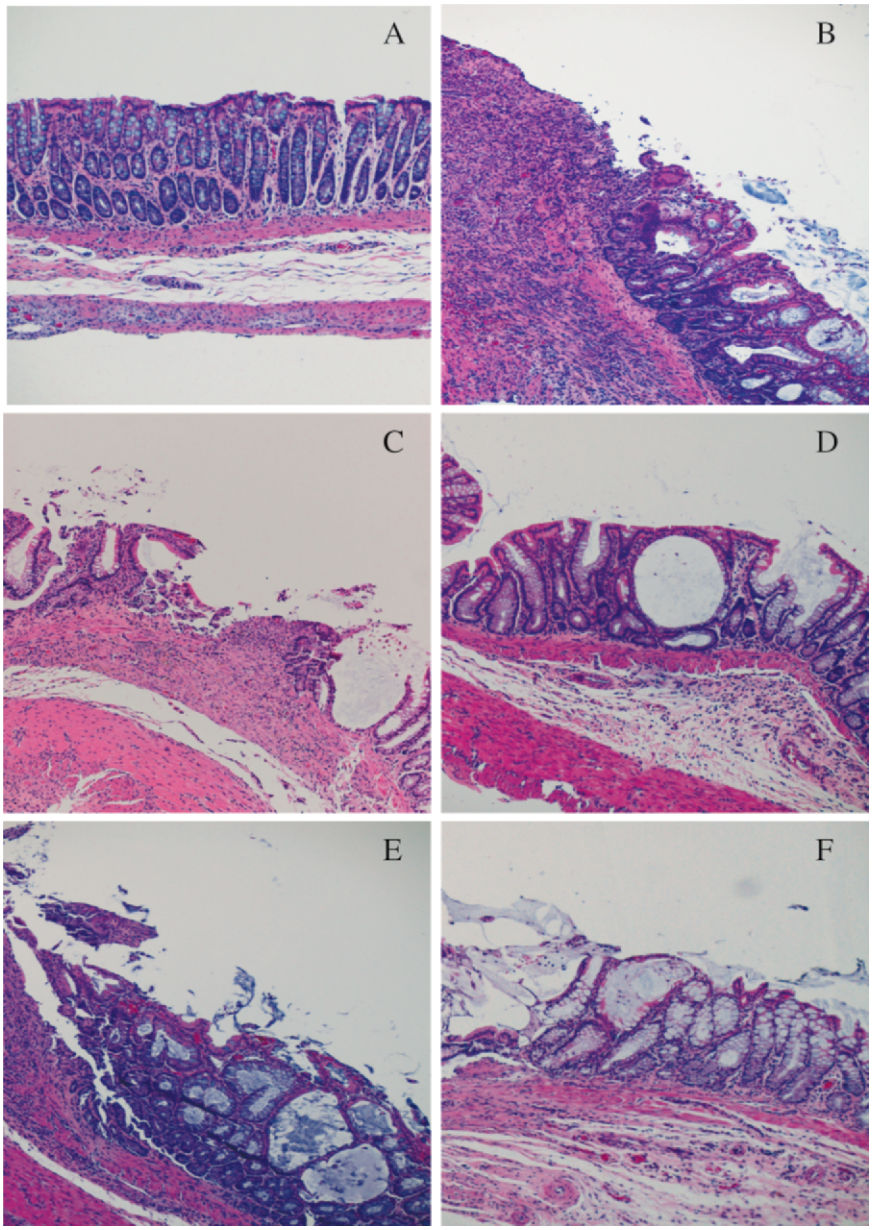

Figure 4. Colonic histological specimens from animals that received intracolonic dosing with A) saline control, B) TNBS, C) TNBS; 5-ASA alone, D) TNBS; ascorbic acid (AA) + 5-ASA, E) TNBS; PBN+ 5-ASA, F) TNBS; α-tocopherol (α-T) + 5-ASA.

plus 5-ASA (Fig. 4D) revealed an absence of mucosal ulceration, the presence of goblet cells, cystic glandular dilatation and evidence of glandular branching. Mild inflammation was present in the lamina propria. PBN plus 5-ASA treatment (Fig. 4E) of TNBS colitis was associated with focal mucosal ulceration, marked cystic glandular dilatation containing mucin-like material and moderate inflammation. Topical therapy with α-tocopherol plus 5-ASA (Fig. 4F) for TNBS colitis resulted in mucosal healing with an absence of mucosal ulceration, replenishment of goblet cells, cystic glandular dilatation with mucin-like material within the lumen, branching within the glandular crypts and minimal inflammation. These histological samples reveal a spectrum of disease activity in response to luminal topical therapies for TNBS colitis. Monotherapy with 5-ASA and combination therapies with specific antioxidants plus 5-ASA showed varying degrees of mucosal repair and reduction in inflammation. In general, the microscopic features of colitis illustrated in these photomicrographs correlate with quantitative measures of colitis described in Figures 2 and 3.

Effects of Antioxidant Plus 5-ASA Therapy on Mucosal Inflammation. MPO activity in the distal colon (Fig. 5) was decreased significantly in response to monotherapy with 5-ASA alone (354 ± 81 ng/gm; $P < 0.05$) when compared to TNBS-induced colitis (703 ± 130 ng/gm). Furthermore, combination therapy with α-tocopherol plus 5-ASA caused reduction in the MPO activity (118 ±

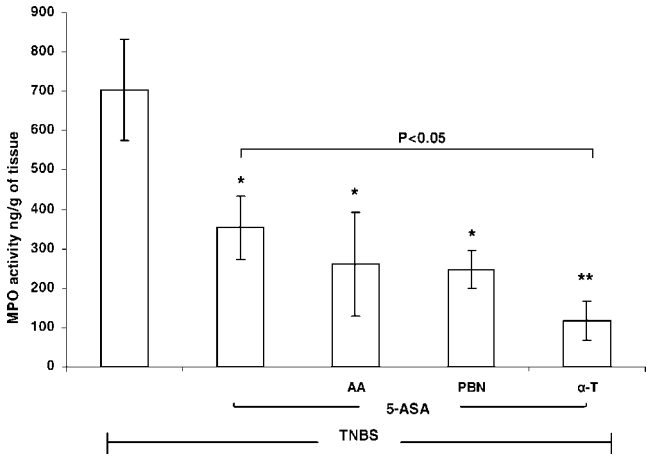

Figure 5. Myeloperoxidase activity in distal colon tissues from animals 11 days after TNBS and 8 days of treatment with: saline, 5-ASA alone, ascorbic acid (AA) + 5-ASA, PBN and α-tocopherol (α-T) + 5-ASA. * $P < 0.05$, ** $P < 0.01$ vs TNBS.

51 ng/gm) that was significantly less than TNBS ($P < 0.01$) and TNBS plus 5-ASA ($P < 0.05$). In contrast, luminal therapies ascorbic acid and PBN plus 5-ASA reduced MPO activity significantly in TNBS colitis ($P < 0.05$) but results were not different from those observed with 5-ASA alone ($P > 0.1$).

Discussion

Based on our previous studies with the antioxidant NAC plus 5-ASA as topical therapy for TNBS colitis (20, 21), we sought to determine if other antioxidants when combined with mesalamine would also exert significant enhancement in the anti-inflammatory effects of 5-ASA. Results of the present studies indicated that, of the antioxidants studied, only α-tocopherol caused significant and synergistic reductions in global and individual indices of microscopic colitis and colonic MPO activity. Ascorbic acid in combination with 5-ASA also decreased significantly the cumulative microscopic injury score and the subset analysis for ulceration but did not significantly reduce other measures of inflammation. Furthermore, the level of MPO activity observed with ascorbic acid plus 5-ASA was significantly less than that recorded for TNBS but was not different from the effect of 5-ASA alone. Inclusion of PBN with 5-ASA in the enema formulation had no significant effect over 5-ASA alone in altering microscopic colitis scores or MPO activity in TNBS colitis. The noteworthy findings of this study are that α-tocopherol and, to a lesser degree, ascorbic acid plus 5-ASA acted topically in rats afflicted with immune-mediated colitis to promote reduction in inflammation and mucosal healing to a degree that was significantly greater than mesalamine alone.

These studies were not designed to examine specific cellular or humoral mechanisms by which these antioxidants might affect the inflammatory and reparative processes in TNBS colitis. The results presented with regard to α-tocopherol, however, demonstrate similarities to previous studies with NAC plus 5-ASA (20, 21) in the terms of the magnitudes of reduction in cumulative and subset parameters of microscopic colitis and MPO activity. Specifically, NAC plus 5-ASA and α-tocopherol plus 5-ASA each reduced the global TNBS colitis injury score to levels that were significantly less than those recorded for 5-ASA alone. Furthermore, subset analysis of individual indices of colitis demonstrated that both NAC plus 5-ASA and α-tocopherol plus 5-ASA reduced significantly each parameter of microscopic colitis. In addition, MPO activity as a biomarker of polymorphonuclear (PMN) leukocyte infiltration in the colon was reduced by greater than 80% with each combination therapy. These findings paralleled the dramatic reductions in PMN numbers in the lamina propria assessed quantitatively by microscopy. It is also worth noting that the antioxidant ascorbic acid when added to 5-ASA enema preparation also caused significant enhancement in the anti-inflammatory actions of mesalamine. These effects, however, were not as pronounced as those observed with α-tocopherol plus 5-ASA. In contrast, the addition of PBN to the 5-ASA formulation was not different than the effects observed with 5-ASA alone. Taken together, the present and previous studies from our laboratory suggest that the addition of antioxidant agents to topically applied mesalamine can significantly augment the anti-inflammatory and healing processes in the treatment of colitis in this experimental model.

It is likely, although not proven, that the combined effects of α-tocopherol and 5-ASA result in reduction in the elaboration of proinflammatory cytokines evoked by TNBS immune-mediated colitis. An indirect indicator of immune mediator down regulation is the profound decrease in mononuclear cell infiltrate observed in the distal colon with α-tocopherol plus 5-ASA treatment. In similarly designed studies, NAC plus 5-ASA inhibited cytokine gene expression elicited by TNBS in the rat colon (20). Among the cytokines examined (IL-1A, IL-1B, IL-4, IL-6 and TNFα) gene expression was inhibited significantly by NAC plus 5-ASA, whereas the results with either NAC or 5-ASA as single agents did not achieve this degree of statistical distinction. Furthermore, the TNBS-associated marked increases in colonic levels of PGE_2 and NO were counteracted by treatment with NAC plus 5-ASA (21). These findings suggest that combination therapy with NAC plus 5-ASA may disrupt a critical link between the proinflammatory effects of PGE_2 and oxidative stress in this model of inflammatory bowel disease.

The administration of a variety of antioxidants by oral, parenteral and intracolonic routes has, in general, been shown to reduce colonic inflammation induced by agents such as DSS and TNBS (13–19). The unique aspect of the present report is that each of the agents with antioxidant properties was administered topically with mesalamine by enema to the distal colon of rats with acute TNBS colitis. α-Tocopherol, as a member of the vitamin E family, possesses

both antioxidant and non-antioxidant properties (22, 23). Although there have been no reported comparable studies on the topical effects of α-tocopherol in experimental colitis the article by Isozaki *et al*. (25) has relevance to the present studies. The authors examined the effects of a water-soluble vitamin E derivative, administered by intraperitoneal injection for 7 days, on TNBS colitis in rats. They observed that this therapy significantly reduced colonic inflammation, MPO activity, cytokine production and lipid peroxidation. In the present study α-tocopherol was solubilized with DMSO and co-administered with mesalamine by enema for 8 days beginning 3 days after induction of TNBS colitis. It is of note that the overall colitis damage score reported by Isozaki *et al*. (25) was reduced by approximately 60% with ip administration of the water-soluble tocopherol derivative while the present study observed 82% decrease in global microscopic colitis with rectally dosed α-tocopherol plus mesalamine. Furthermore, MPO activity in the Isozaki *et al*. (25) study was maximally reduced by 43% while topical treatment with α-tocopherol plus mesalamine, in the present study, decreased colonic MPO activity by 83%. Thus, both studies describe a beneficial therapeutic effect of α-tocopherol in TNBS colitis and suggest that the antioxidant properties of this vitamin, whether administered systemically or topically, contribute to the observed reduction in colonic inflammation. A limitation of the present studies was that the study design did not allow for the assessment of the effects of topical α-tocopherol alone in the treatment of TNBS colitis.

Phenyl butylnitrone (PBN) is a spin-trapping agent capable of free radical scavenging and conferring cellular protection in a number of disease states including ischemia-reperfusion injury (19). Intraperitoneal administration of PBN has been shown to protect against DSS-induced colitis in mice (19). The authors of this study observed that PBN treatment inhibited colonic activation of NF-kB and down regulated mRNA expression of TNF-α, IFN-α and iNOS. This study confirms that PBN, like other antioxidants, including α-tocopherol, ascorbic acid, N-acetylcysteine and mesalamine, can inhibit NF-kB activation (11, 19, 24, 26, 27). The present study recorded that PBN plus 5-ASA was comparable to, but not better than, 5-ASA alone in reducing TNBS-induced microscopic features of colonic inflammation and MPO activity. Furthermore, macroscopic evidence of TNBS colitis was not affected by PBN plus 5-ASA. An explanation for the discrepancy between the macroscopic and microscopic findings of colitis with PBN plus 5-ASA is not apparent. Although the cumulative histological colitis score observed with PBN plus 5-ASA was significantly less than that recorded for TNBS alone (Fig. 2) the subset assessment of ulceration was increased relative to 5-ASA monotherapy (Fig. 3). It is possible that PBN plus 5-ASA may exert a net pro-oxidant effect and, thus, compromise the therapeutic action of this combination therapy (23, 26). In contrast to the study by Naito *et al*. (19), PBN in the present study was administered rectally at a single dose (30 mg/kg) with 5-ASA and not as a sole agent. In addition, dose ranging studies with PBN either in the presence or absence of 5-ASA were not performed. Owing to these constraints in experimental design it is possible that we may not have fully appreciated the effectiveness of PBN as an antioxidant/anti-inflammatory substance in these experiments.

Ascorbic acid is water-soluble and absorbed in the small intestinal and colonic cells by ascorbate-specific transporters (28–31). The antioxidant properties of ascorbic acid are enhanced by its ability to recycle glutathione and vitamin E (32). The antioxidant buffering capacity and defense systems within colonic epithelium have been shown to be impaired under clinical and experimental states of inflammation (33, 34). The novel applications of ascorbic acid plus 5-ASA, as topical treatment for TNBS colitis in the present study, has demonstrated superiority of this combination therapy versus 5-ASA alone in reducing histological and biomarker (MPO activity) measures of colitis. Results of our studies with α-tocopherol and ascorbic acid indicate that inclusion of these antioxidants with mesalamine significantly enhances the pharmacological effectiveness of 5-ASA in topical treatment of TNBS colitis.

In summary, the central finding of this study is evidence that antioxidant substances when combined with 5-ASA in an enema formulation augment significantly the anti-inflammatory actions of mesalamine in the treatment of TNBS colitis. We have previously shown that the combination of N-acetylcysteine plus 5-ASA synergistically enhanced reduction in inflammation and acceleration of mucosal healing in this model of experimental colitis when compared to 5-ASA alone. The present study extends these observations to the antioxidants α-tocopherol and ascorbic acid, as co-agents with mesalamine, when applied as topical luminal therapy, for chemically induced colitis. The principles established in these studies create the foundation to further investigate antioxidant/anti-inflammatory drug combination therapies that may have application to the treatment of inflammatory bowel disease.

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