# Superoxide dismutase ameliorates TNBS-induced colitis by reducing oxidative stress, adhesion molecule expression, and leukocyte recruitment into the inflamed intestine

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**Abstract: Oxidant stress has been implicated in the pathogenesis of inflammatory bowel disease. Antioxidant enzymes, such as superoxide dismutase (SOD), are candidate drugs for modulating this pathogenic factor. This study was designed to determine the therapeutic value of SOD in an experimental model of colitis and to study the mechanisms underlying its effects on intestinal inflammation. For that purpose, colitic (trinitrobenzene sulfonic acid-induced) and control rats were studied. Groups of colitic animals were treated with different doses of SOD (1, 4, or 13 mg/kg/day) or vehicle, starting after induction of colitis and during 7 days. Clinical and pathological markers of colitis severity and lipid peroxidation in colonic tissue were measured. Leukocyte-endothelial cell interactions in colonic venules and expression of vascular cell adhesion molecule 1 (VCAM-1) were determined. Development of colitis was associated with a significant loss in body weight, an increase in macroscopic and microscopic damage scores, and colonic myeloperoxidase activity. Administration of SOD significantly attenuated these changes in a dose-dependent manner and reduced lipid peroxidation in colonic tissue. The increase in leukocyte rolling and adhesion in colonic venules of colitic rats were significantly reduced by administration of SOD, 13 mg/kg/day. Development of colitis was associated with a marked increase in endothelial VCAM-1 expression, which was significantly reduced by treatment with SOD. In conclusion, treatment with SOD significantly reduces peroxidation reactions in the inflamed colon and affords significant amelioration of colonic inflammatory changes in experimental colitis. This effect is related to a reduction in VCAM-1 expression and leukocyte recruitment into the inflamed intestine. *J. Leukoc. Biol.* 76: 537–544; 2004.**

***Key Words:*** *inflammatory bowel disease · reactive oxygen species · vascular cell adhesion molecule-1*

## INTRODUCTION

Several factors are recognized to contribute to the pathogenesis of inflammatory bowel disease (IBD) including an overgeneration of reactive oxygen species (ROS) [1, 2]. Previous studies demonstrated that peripheral blood monocytes [3] and isolated intestinal macrophages from patients with IBD produce free radicals [4]. Also, high numbers of peripheral neutrophils, which are capable of producing large amounts of superoxide, migrate into the intestinal wall in active IBD [1]. A growing body of evidence indicates that ROS, such as peroxide anion, hydrogen peroxide ($H_2O_2$), and hypochlorus acid, are not merely byproducts of the inflammatory process, but they are actually involved in its pathogenesis. To regulate overall ROS levels, the intestinal mucosa possesses a complex of antioxidant systems, of which the superoxide dismutases (SOD) are the initial enzymes, converting superoxide anion to $H_2O_2$. SOD expression in patients with active IBD seems to be altered. In particular, decreased protein activity and levels of cytoplasmic Cu/Zn-SOD have been reported consistently [5–7].

Several experimental strategies have been used to address the importance of the enhanced production of superoxide in the pathogenesis or IBD, but inconsistent findings have left this issue largely unresolved. For example, some reports have described a beneficial effect of SOD treatment in the prevention of experimental colitis [8, 9] and of a SOD mimetic in the treatment of established colitis [10], whereas in studies using transgenic mice overexpressing SOD, a more severe colitis [11] or a reduction in neutrophil infiltration without affecting the clinical or histological severity of colitis has been reported [12]. Therefore, further investigation about the effects of SOD on IBD seems warranted, especially elucidating the value of this therapeutic approach in established colitis.

One of the key aspects in the pathogenesis of IBD that contributes to perpetuate and amplify the inflammatory damage is the recruitment of inflammatory cells into the inflamed intestine. Adhesion of circulating leukocytes to intestinal en-

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Received March 25, 2004; revised May 7, 2004; accepted May 9, 2004; doi: 10.1189/jlb.0304196.

dothelium depends on the coordinated expression of adhesion molecules on endothelial cells, including vascular cell adhesion molecule 1 (VCAM-1), mucosal addressin cell adhesion molecule 1 (MAdCAM-1), and intercellular adhesion molecule 1 (ICAM-1), along with their respective integrin counter-receptors expressed on the surface of circulating leukocytes [13]. The fundamental importance of increased VCAM-1 expression in IBD in particular is supported by reports demonstrating that leukocyte recruitment and mucosal damage in experimental models of colitis are blocked by immunoneutralization of this adhesion molecule [14, 15]. Direct comparison of the relative efficacy of ICAM-1, MAdCAM-1, and VCAM-1 immunoneutralization in the treatment of experimental colitis showed that the latter was the most effective strategy [15]. It has been shown recently that immunoneutralization of $\alpha 4$ integrins, the receptors for VCAM-1 and MAdCAM-1, is effective in the treatment of human Crohn's disease, as its efficacy is similar to that of anti-tumor necrosis factor (TNF)-blocking strategies [16, 17].

ROS induce a quantitative up-regulation of VCAM-1 by de novo synthesis. Functional analysis of the human VCAM-1 promoter demonstrated that several oxidant-sensitive transcription factors are associated with activation of VCAM-1 gene expression in response to interleukin (IL)-1$\beta$ and TNF-$\alpha$ [18, 19]. Furthermore, in vitro studies have shown that expression of SOD but not catalase by adenovirus suppressed TNF-$\alpha$-induced VCAM-1 mRNA accumulation [18].

Based on these observations, the present study was designed to evaluate whether treatment with SOD exerts protection on established experimental colitis induced by trinitrobenzene sulfonic acid (TNBS) and if so, highlight possible mechanisms through which SOD may confer protection, specifically its effects on adhesion molecule expression and leukocyte recruitment.

## MATERIALS AND METHODS

### Induction of colitis

Male Sprague-Dawley rats weighing 300–350 g were obtained from CRIFFA, S.A. (Santa Perpètua de la Mogoda, Spain). Colitis was induced by intracolonic administration of 30 mg TNBS (Sigma Química, Madrid, Spain) in 0.25 mL 50% (vol/vol) ethanol (Merck, Darmstadt, Germany) [20]. Control rats received 0.25 mL saline. Principles of laboratory animal care (National Institutes of Health, publication no. 86-23, revised 1985) and the guidelines of procedures for animal experiments from the Generalitat de Catalunya were followed.

### Treatment groups

Groups of colitic animals were treated with different daily subcutaneous (s.c.) doses of Cu/Zn SOD: 1 mg/kg/day, 4 mg/kg/day, or 13 mg/kg/day or vehicle (saline). For the current study, a preparation of SOD commercially available (Ontosein®, Tedec-Meiji Farma Laboratories, Alcalá de Henares, Spain) was used. The doses of SOD used are based on previous evidence showing that treatment with SOD, 4 mg/kg for 3 days, is effective in reducing leukocyte recruitment in irradiation-induced, intestinal inflammation [21] and a beneficial effect of treatment with 13 mg/kg SOD in reducing pulmonary inflammation associated with severe acute pancreatitis [22]. The first injection of SOD was administered 4 h after the induction of colitis. This time-point was chosen based on previous data from our group showing that intracolonic TNBS induces a progressive depletion of reduced glutathione from 0.5 h to 4 h, and this early depletion may contribute to the initiation of intestinal inflammation in this model [23]. Treatment was administered once daily up to the end of the study at day 7.

### Assessment of colonic inflammatory changes

Animals were weighed daily. Animal groups were studied 7 days after induction of colitis to assess macroscopic, histological, and biochemical colonic changes; the number of animals studied in each experimental group was as follows: controls, n = 6; colitis treated with vehicle, n = 6; colitis treated with SOD, 1 mg/kg/day, n = 6; colitis treated with SOD, 4 mg/kg/day, n = 7; colitis treated with SOD, 13 mg/kg/day, n = 8. To determine the early effects of SOD, additional groups of colitic rats treated with vehicle (n=6) or SOD 13 mg/kg/day (n=6) were studied 24 h after induction of colitis. Anesthesia was induced by administration of thiobutabarbital (Inactin®, Research Biochemicals International, Natick, MA), 100 mg/kg body weight intraperitoneally (i.p.). Samples of blood were collected by open cardiac puncture under aseptic conditions using a 1-ml syringe and placed in Eppendorf vials; blood was spun at 1500 g for 10 min at 4ºC; the supernatant serum was then pipetted into autoclaved Eppendorf vials and frozen at −80ºC for later assay of IL-6 levels using a quantitative, colorimetric commercial kit (R&D Systems, Barcelona, Spain). Thereafter, the colon was excised, opened by a longitudinal incision, rinsed with saline, and weighed (8 cm distal colon). Macroscopic damage was assessed by a single observer blinded to characteristics or treatment of the animal being studied, according to a previously established score that takes into account presence of adhesions (score 0–2), strictures (score 0–3), ulcers (score 0–3), and wall thickness (score 0–2) [24]. Two colon samples (150–200 mg) were then excised, snap-frozen in liquid nitrogen, and stored at −80°C for later assay of myeloperoxidase (MPO) activity and lipid hydroperoxide (LPO) concentration. Additional samples were preserved in 10% formalin for histologic studies. For assessment of microscopic damage, formalin-fixed colonic samples were embedded in paraffin, and sections (5-$\mu$m thick) were stained with hematoxilin-eosin (H&E). The degree of inflammation of the colon was graded semiquantitatively from 0 to 11 according to previously defined criteria [25].

### MPO assay

MPO activity was measured photometrically by using 3,3′,5,5′-tetramethylbenzidine as a substrate [26]. Samples were homogenized with 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0. Homogenates were then disrupted for 30 s by using a Labsonic (Braun Biotech, Melsungen, Germany) sonicator and subsequently snap-frozen in dry ice and thawed on three consecutive occasions before a final 30-s sonication. Samples were incubated at 60°C for 2 h and then spun down at 4000 *g* for 12 min. Supernatants were collected for MPO assay. Enzyme activity was assessed photometrically at 630 nm. The assay mixture consisted of 20 mL supernatant, 10 mL tetramethylbenzidine (final concentration, 1.6 mM) dissolved in dimethyl sulfoxide, and 70 mL $H_2O_2$ (final concentration, 3.0 mM) diluted in 80 mM phosphate buffer, pH 5.4. An enzyme unit is defined as the amount of enzyme that produces an increase of 1 absorbance unit per minute.

### Measurement of lipid peroxidation

Quantification of lipid peroxidation was performed in colon tissue samples (150–200 mg) by direct measurement of LPO according to a previously described technique [27], based on the property of hydroperoxides to readily react with ferrous ions to produce ferric ions, which are detected using thiocyanate ions as the chromogen. In brief, after tissue samples were homogenized, LPO were extracted into chloroform and stored at −80ºC until the time of the assay. For performing the assay, the commercially available kit from Cayman Chemical (Ann Arbor, MI) was used following the manufacturer's instructions. Results are expressed in nmols LPO/g tissue.

### Endothelial VCAM-1 expression

Thirty rats were used to characterize endothelial expression of VCAM-1. Six control (saline-treated) rats and 24 animals with TNBS colitis treated with vehicle or SOD, 1 mg/kg, 4 mg/kg, or 13 mg/kg (n=6 per group) were studied.

The monoclonal antibodies (mAb) used were 5F10, a murine immunoglobulin $G_{2a}$ ($IgG_{2a}$) against rat VCAM-1 [28], and UPC-10, a nonbonding, murine $IgG_{2a}$. 5F10 was obtained from Biogen Inc. (Cambridge, MA) and UPC-10, from Sigma Química.The binding mAb 5F10 directed against VCAM-1 was

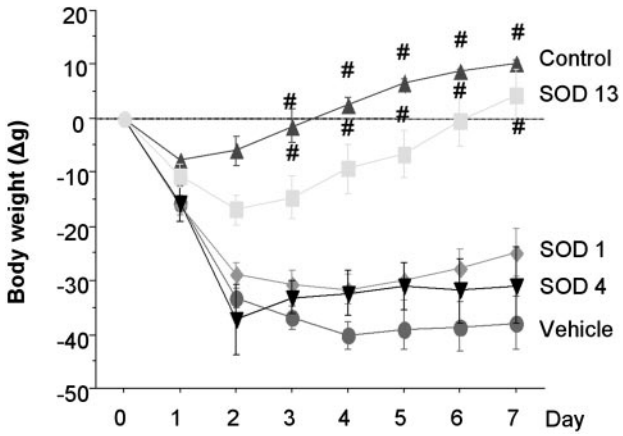


**Fig. 1.** Effect of treatment with various doses of SOD (1, 4, or 13 mg/kg) or vehicle on daily changes in body weight loss in colitic animals. #, $P < 0.05$, versus SOD, 1 mg/kg; SOD, 4 mg/kg; and vehicle.

labeled with $^{125}$I, whereas the nonbinding mAb UPC-10 was labeled with $^{131}$I (Amersham Ibérica, Madrid, Spain). Radioiodination of the mAb was performed by using the iodogen method as described previously [29].

Animals were anesthetized with thiobutabarbital (100 mg/kg body weight i.p.), and the right carotid artery and right jugular vein were cannulated. For assessment of endothelial VCAM-1 expression, 20 μg $^{125}$I-5F10 was used. In all cases, 5 μg $^{131}$I-UPC-10 was added to the mixture. Doses of anti-VCAM-1 mAb proved to be saturating in previous assays [14]. The mixture of binding and nonbinding mAb was administered through the jugular catheter. The injected activity in each experiment was calculated by counting a 5-μL sample of the mixture containing the radiolabeled mAb. The accumulated activity of each mAb in an organ was expressed as nanograms of binding antibody per gram of tissue. The formula used to calculate VCAM-1 expression was as follows: endothelial expression = [(cpm $^{125}$I organ·g$^{-1}$·cpm $^{125}$I-injected$^{-1}$)–(cpm $^{131}$I organ·g$^{-1}$·cpm $^{131}$I-injected$^{-1}$)×(cpm $^{125}$I in plasma)/(cpm $^{131}$I in plasma)]·ng-injected mAb. This formula was modified from the original method [30] to correct the tissue accumulation of nonbinding mAb for the relative plasma levels of binding and nonbinding mAb [31].

## In vivo assessment of leukocyte-endothelial cell interactions in colonic venules

Leukocyte-endothelial cell interactions in colonic submucosal (s.m.) and lamina propria venules were characterized by using intravital microscopic techniques in additional groups of control animals and in rats with TNBS-induced colitis treated with vehicle; SOD, 1 mg/kg; SOD, 4 mg/kg; or SOD, 13 mg/kg (n=5–7 animals per group). These studies were also performed at day 7 after induction of colitis. Rats were anesthetized with thiobutabarbital (100 mg/kg body weight i.p.), the right carotid artery was cannulated, and the abdomen was opened via a midline incision. A segment of the distal colon was chosen for microscopic examination, gently exteriorized, and covered with a cotton gauze soaked with bicarbonate buffer. Rats were then placed on an adjustable microscope stage, and the colon was extended over a nonautofluorescent coverslip that allowed observation of a 2-cm$^2$ segment of tissue. An inverted microscope (Diaphot 300, Nikon, Tokyo, Japan) with a CF Fluor 40× objective lens (Nikon) was used. A charge-coupled device (CCD) camera (model XC-77, Hamamatsu Photonics, Japan) with a C2400 CCD camera control unit and a C2400-68 intensifier head mounted on the microscope projected the image onto a monitor (Trinitron KX-14CP1, Sony, Tokyo, Japan). The images were recorded using a videocassette recorder (SR-S368E, JVC, Tokyo, Japan). Leukocytes were in vivo-labeled by s.c. injection of rhodamine 6G (Molecular Probes, Leiden, The Netherlands). The route of administration was changed relative to previous methodology [14] after observing that the s.c. route produces a more even and sustained leukocyte fluorescence. Rhodamine 6G-associated fluorescence was visualized by epi-illumination at 510–560 nm, using a 590-nm emission filter. Single unbranched, s.m. and lamina propria venules ranging between 15 and 25 μm in diameter (D) were studied. The flux of rolling leukocytes, number of rolling leukocytes, and number of adherent leukocytes in 100 μm venule were determined off-line after playback of videotapes, as described previously [14]. Venular blood flow (Vbf) was estimated from the mean of the velocity of three free-flowing leukocytes (ffv), using the empirical relationship of Vbf = ffv/1.6 [32]. Venular wall shear rate ($\gamma$) was calculated, assuming cylindrical geometry, using the Newtonian definition, $\gamma = 8$ (Vbf/D) [33]. In each animal, three to six venules were examined, and values for number of rolling and adherent leukocytes, leukocyte rolling velocity, and venular wall shear rate were calculated as the mean of each parameter in all venules examined.

## Statistical methods

Data were analyzed using analysis of variance with Bonferroni (post-hoc) test or the Kruskal-Wallis test with Dunn's multiple comparison test when appropriate. Values are expressed as mean ± SE. Statistical significance was set at $P < 0.05$.

# RESULTS

## Effects of treatment with SOD on TNBS-induced colitis

Treatment with SOD at the dose 13 mg/kg per day significantly attenuated the decrease in body weight from 3 days after initiation of treatment up to the end of the study relative to animals receiving vehicle or treatment with 1 or 4 mg/kg/day SOD. In addition, animals treated with SOD, 13/mg/kg/day, rapidly regained weight, whereas in those receiving vehicle or SOD at lower doses, the decrease in body weight persisted for the 7 days of the study (**Fig. 1**).

Treatment with SOD, 13 mg/kg, also resulted in a significant reduction of the macroscopic damage score at day 7 compared with animals receiving vehicle or SOD at doses of 1 or 4 mg/kg/day (**Table 1**). Concurrent with these findings, colon weight was also significantly reduced in colitic animals treated with the highest dose of SOD (Table 1). Histological damage score was also significantly reduced by treatment with 13 mg/kg/day of SOD (Table 1). In particular, animals treated with 13 mg/mg SOD had a marked reduction of the inflammatory infiltrate that was mostly limited to the mucosa, without surpassing the lamina propria, and extensive areas of ulcerations

TABLE 1. Effects of SOD Treatment on Clinical, Pathologic, and Biochemical Markers of Colitis Severity

| | Control n = 6 | Vehicle n = 6 | SOD, 1 mg/kg n = 6 | SOD, 4 mg/kg n = 7 | SOD, 13 mg/kg n = 8 |
|---|---|---|---|---|---|
| Macroscopic score | 0.0 ± 0.0 | 7.3 ± 0.8* | 7.7 ± 0.6* | 7.5 ± 0.8* | 4.1 ± 0.7*,† |
| Histologic score | 0.0 ± 0.0 | 8.6 ± 0.5* | 7.3 ± 0.3* | 8.3 ± 0.7* | 5.6 ± 0.6*,† |
| Distal colon weight (g) | 0.6 ± 0.1 | 4.3 ± 0.4* | 4.8 ± 0.6* | 4.1 ± 0.7* | 2.1 ± 0.3† |
| IL-6 (pg/ml) | 51.9 ± 27.2 | 210.8 ± 64.5* | 204.8 ± 20.1* | 94.2 ± 14.6† | 108.6 ± 14.4† |

* $P < 0.05$ versus control; † $P < 0.05$ versus vehicle.

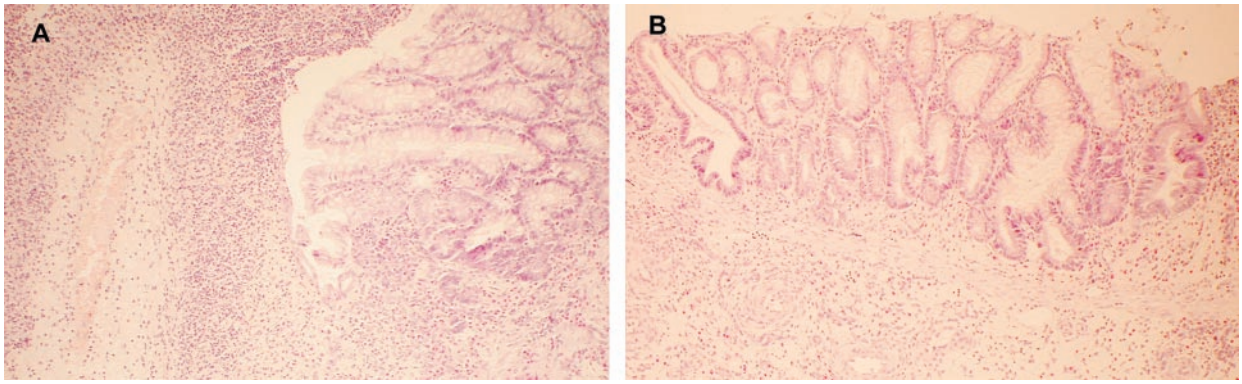


**Fig. 2.** Representative histologic lesions of colitic rats treated with vehicle (A) or SOD, 13 mg/kg/day (B), for 7 days. An intense, inflammatory infiltrate in the mucosa and s.m. with extensive areas of ulceration was seen uniformely in vehicle-treated, colitic rats. By contrast, SOD-treated rats and a marked reduction of the inflammatory infiltrate, mostly limited to the lamina propria, and minimal ulceration. A distortion of crypt architecture is still evident in the latter group. H&E, 100×.

characteristic of the vehicle-treated animals were not observed in the SOD-treated group (**Fig. 2**). Neutrophil infiltration into the inflamed intestine, measured as MPO activity, was significantly reduced by treatment with SOD. Of note, treatments with 4 and 13 mg/kg/day SOD were followed by significant reductions of MPO activity (**Fig. 3**).

To rule-out the possibility that treatment with SOD, started at 4 h after induction of colitis, was exerting its beneficial effect by preventing the inducing action of TNBS, additional groups of animals were studied at 24 h. At this time-point, in which no differences in body weight were detected as shown in Figure 1, animals treated with vehicle or with SOD, 13 mg/kg/day, had a similar macroscopic colitis score (4.3±0.3 vs. 4.0±0.0), histological colitis score (7.3±0.3 vs. 6.8±0.6), and distal colon weight (2.1±0.2 g vs. 2.4±0.3 g).

Development of TNBS-induced colitis was associated with a significant increase of IL-6 levels in plasma. As shown in Table 1, treatment with SOD at doses of 4 and 13 mg/kg/day significantly reduced the increase in IL-6 concentrations in plasma relative to colitic animals receiving vehicle or 1 mg/kg/day SOD.

## Effects of treatment with SOD on colonic lipid oxidation

Levels of LPO in colonic tissue from different experimental groups are shown in **Figure 4**. In comparison with control animals, development of colitis was associated with a significant increase in LPO in colonic tissue (0.5±0.4 nmol/g vs. 9.2±2.8 nmol/g, $P<0.05$). Treatment with SOD, 1 mg/kg/day, for 1 week, which did not alter the severity of colitis, did not induce any significant changes in the levels of LPO in colon (8.1±1.4 nmol/g) when compared with vehicle-treated animals. The levels of LPO were significantly lower in colitic animals treated with 4 mg/kg (3.4±1.6 nmol/g) or 13 mg/kg (2.7±1.6 nmol/g) of SOD; these levels were not significantly different from the noncolitic group.

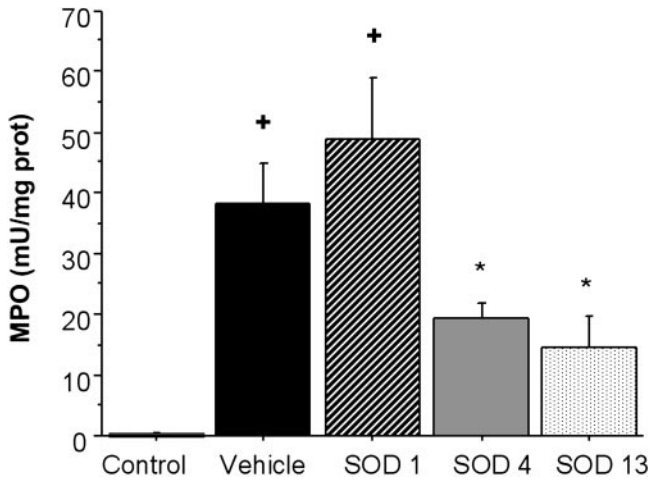


**Fig. 3.** Effect of treatment with vehicle or different doses of SOD (1, 4, or 13 mg/kg) on colonic MPO activity in animals studied 7 days after induction of colitis. +, $P < 0.05$, versus control animals; *, $P < 0.05$, versus vehicle-treated, colitic animals.

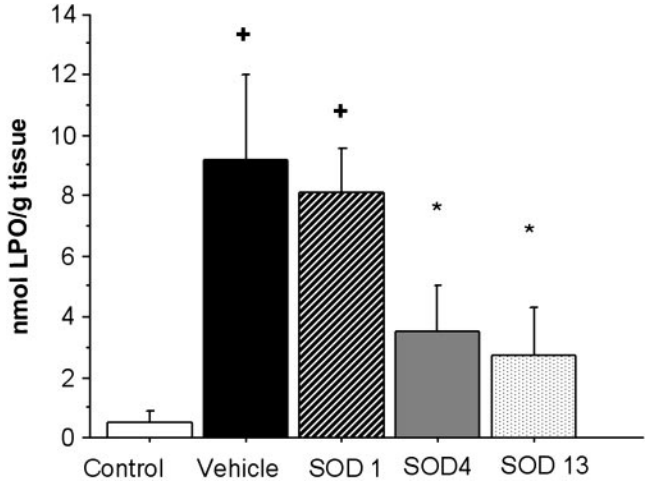


**Fig. 4.** Effects of treatment with vehicle or different doses of SOD (1, 4, or 13 mg/kg) on concentration of lipid peroxides in colitic animals studied 7 days after induction of colitis. +, $P < 0.05$, versus controls; *, $P < 0.05$, versus vehicle-treated, colitic animals.

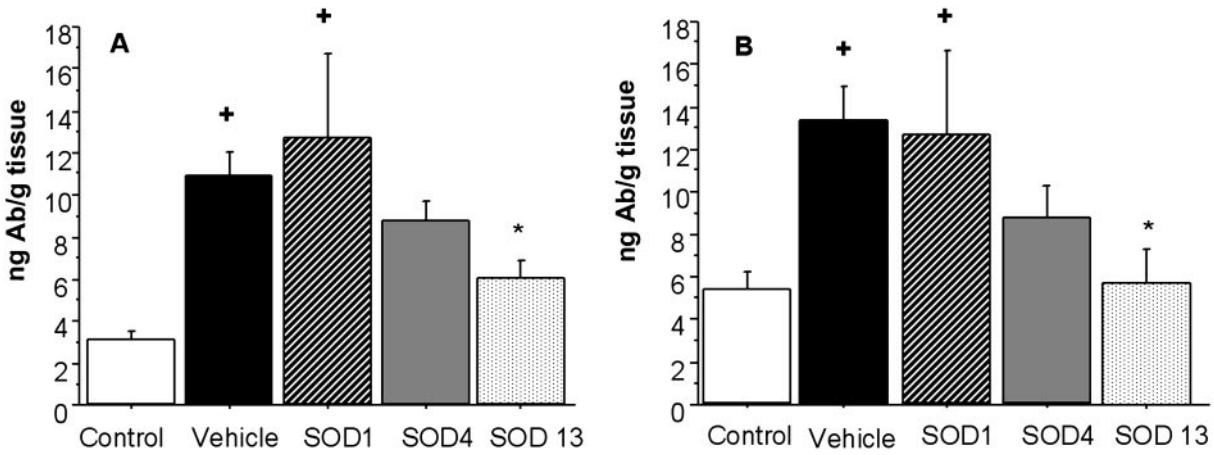


**Fig. 5.** Expression of endothelial VCAM-1 in (A) colon, (B) cecum relative to tissue weight in animals studied 7 days after induction of colitis. Effects of treatment with SOD. +, $P < 0.05$, versus controls; *, $P < 0.05$, versus vehicle-treated, colitic animals. Ab, Antibody.

## Effects of treatment with SOD on VCAM-1 expression

In control animals, expression of VCAM-1 in colonic endothelium was very low. Appearance of colitis was associated with a marked up-regulation of VCAM-1 expression. This increment was dose-dependently attenuated by treatment with SOD, the dose of 1 mg/kg/day had no effect, a moderate but nonsignificant reduction in response to treatment with 4 mg/kg/day, and a significant reduction following treatment with the highest dose (**Fig. 5A**). The pattern of VCAM-1 expression in the more proximal colon (cecum) followed a similar pattern (Fig. 5B).

## Leukocyte-endothelial cell interactions in colonic venules

Colitic rats showed a pronounced increase in leukocyte-endothelial cell interactions compared with control animals. There was a 12-fold increase in the flux of rolling leukocytes 7 days after induction of colitis. Treatment with SOD, 13 mg/kg/day, significantly reduced the flux of rolling leukocytes in colonic venules (**Fig. 6A**). Similar findings were found when the number of rolling leukocytes (rolling cells/100 μm venule) was analyzed (Fig. 6B).

Very few adherent leukocytes were present in venules of control rats. A 16-fold increase in leukocyte adhesion was observed 7 days after induction of colitis in vehicle-treated rats. Treatment with SOD at the doses of 4 mg/kg/day and 13 mg/kg/day for 7 days resulted in a significant reduction in the number of adherent leukocytes, whereas the lower dose, 1 mg/kg/day, of SOD did not significantly modify the number of adherent leukocytes in colonic postcapillary venules (**Fig. 7**).

No differences in venular shear rate were observed between groups of control and colitic rats treated with SOD or vehicle (data not shown). Treatment with SOD did not result in changes in the number of peripheral blood circulating leukocytes (data not shown).

## DISCUSSION

Cu/Zn SOD is an enzyme widely distributed in the cytoplasm of all mammalian cells and has been shown to exert anti-

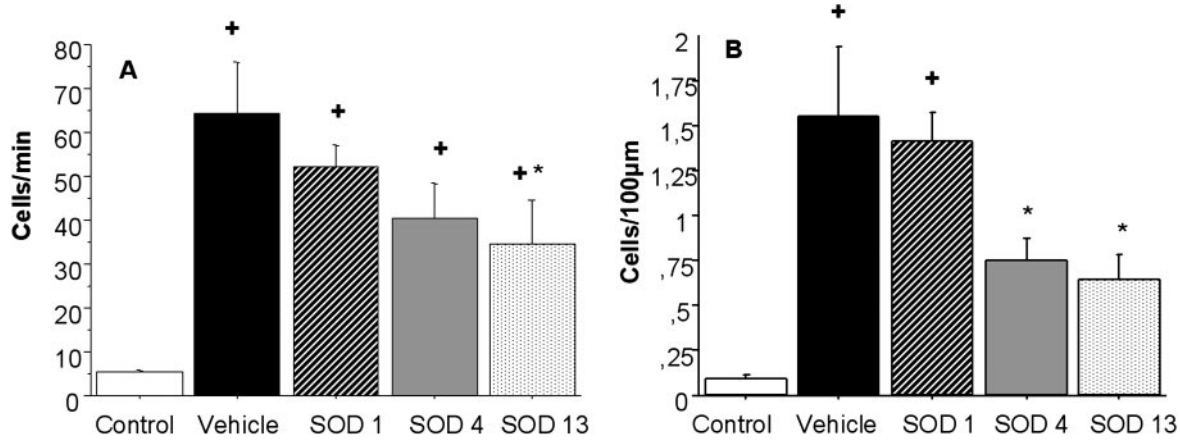


**Fig. 6.** Effects of treatment with vehicle or different doses of SOD on flux of rolling leukocytes (A) and number of rolling leukocytes (B) in colonic venules in animals studied 7 days after induction of colitis. +, $P < 0.05$, versus controls; *, $P < 0.05$, versus vehicle-treated, colitic animals.

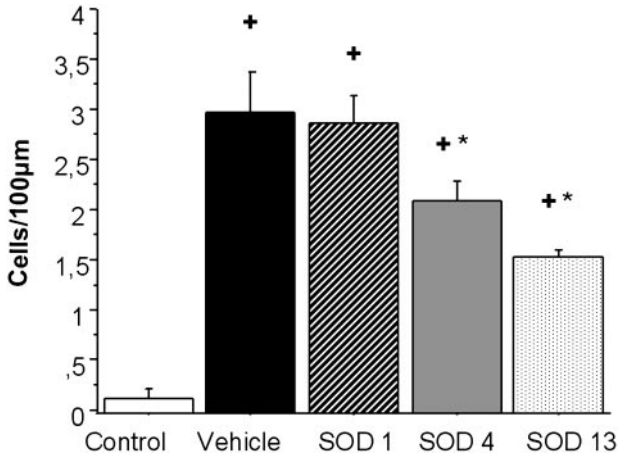


**Fig. 7.** Effects of treatment with vehicle or different doses of SOD on firm leukocyte adhesion in colonic venules in animals studied 7 days after induction of colitis. +, $P < 0.05$, versus controls; *, $P < 0.05$, versus vehicle-treated, colitic animals.

inflammatory effects in a variety of experimental models [9, 22, 34]. Human studies have reported decreased intestinal tissue levels of Cu/Zn SOD in patients with IBD [35], and pilot studies, including a very limited number of patients with ulcerative colitis or Crohn's disease, have reported a clinical response to treatment with Cu/Zn SOD [36], although this treatment is not currently used in the clinical setting. [37]

The current study demonstrates that treatment with Cu/Zn SOD effectively ameliorates TNBS-induced colitis in a dose-dependent manner. The beneficial effects of treatment with SOD, 13 mg/kg/day, are of similar magnitude to those previously reported by our group for dexamethasone in the same model of experimental colitis [38]. In keeping with the current observations, a previous study documented a significant reduction in macroscopic and microscopic scores in TNBS-induced colitis after 7 days of treatment with SOD [39]. However, in this study, the authors noted a significant amelioration in the treated group as early as 24 h after the induction of colitis, which may be related to the initiation of SOD treatment before colitis induction. In the current study, we chose to start treatment after colitis was established based on two main reasons: One is the superior clinical relevance of this strategy, as in the clinical setting, we treat patients with active disease, and a significant proportion of treatments that are useful when used prophylactically proves to be ineffective in established colitis. The second important reason to start treatment after induction of colitis by TNBS is that administering an antioxidant before TNBS might interfere with the process of colitis induction by this agent. Evidence has indicated that some of the inflammation produced by intrarectal administration of TNBS may be mediated by a burst of ROS produced from its metabolism within the mucosal interstitium [40]. Previous studies characterizing these effects over time indicate that most of the toxic effects of TBNS occur within 30 min of administration of this substance, and in a previous study, we observed that there is no further depletion of antioxidant systems in the colon after 4 h of TNBS administration [23]. The group of animals studied 24 h after induction of colitis treated with the highest dose of SOD had inflammatory lesions of similar severity to those of colitic animals treated with vehicle, which excludes a direct effect of SOD hampering the development of colitis in our experimental setting.

Measurements of lipid peroxidation confirm previous evidence, indicating that development of colitis is associated with a significant burst in ROS, and we show that treatment with SOD dose-dependently inhibits peroxidation of lipids in the inflamed intestine. There is an apparent discrepancy between the relatively mild, beneficial effect of treatment with 4 mg/kg/day SOD on parameters of colitis severity and the robust effect of this treatment on lipid peroxidation, decreasing lipid peroxides in the inflamed intestine to levels similar to those observed after treatment with 13 mg/kg/day SOD and not different from controls. This may be related to the fact that peroxidation of lipids is only one of the effects of ROS, and a dose of SOD that significantly affects this process may have weaker effects on other factors more sensitive to oxidative stress such as activation of adhesion molecule expression and the process of leukocyte recruitment.

Recruitment of leukocytes from the circulation to the extravascular space is a critical event in inflammation. Two key factors regulating these complex processes are oxidant stress and specific adhesion molecules. It has previously been shown that modulation of oxidant stress in conditions such as ischemia-reperfusion or irradiation markedly reduces leukocyte trafficking into the affected organ [21, 41]. Our results now extend these observations to the field of IBD. Leukocyte-endothelial cell interactions were attenuated in response to treatment with 4 mg/kg SOD, with a significant reduction of the number of rolling leukocytes and a 30% reduction in the number of firmly adherent leukocytes, although the latter change did not reach statistical significance. This dose of SOD also induced a significant reduction (50%) in MPO activity, which has been shown by others to be proportional to the number of neutrophils in the inflamed tissue [42]. Therefore, recruitment of neutrophils seems to be particularly influenced by antioxidants. This contention is supported by a recent work showing that overexpression of Cu/Zn SOD in transgenic mice significantly reduced MPO activity in the colon without affecting the clinical or histological severity of colitis [12]. The highest dose of SOD tested in the current study (13 mg/kg/day) produced a significant reduction in leukocyte rolling and firm adhesion, and this was associated with a marked amelioration of all parameters of colitis severity.

The initial rolling interactions are mediated by the selectins, whereas firm adhesion and diapedesis are mediated by the interaction of integrins on the surface of leukocytes with endothelial adhesion molecules or the Ig superfamily. In a recent and elegant study, Cuzzocrea et al. [10] demonstrated that treatment with a low molecular weight SOD mimetic significantly reduced expression of P-selectin and ICAM-1 in colonic tissue. Our observation of reduced leukocyte rolling interactions in colonic postcapillary venules of colitic rats in response to treatment with SOD is in line with the notion that this treatment may affect expression of selectins. Nevertheless, the reduced rolling interaction between leukocytes and endothelial

cells in response to treatment with SOD probably is not a key factor in affording a clinical benefit in this model of colitis. We have previously demonstrated that TNBS-induced colitis blockade of E-, P-, or L-selectin, in spite of resulting in significant reductions in rolling interactions in colonic venules, does not alter the course of the disease [38].

Therefore, to further explore the mechanistic basis for the effects of SOD on intestinal inflammation, we concentrated on endothelial cell adhesion molecules involved in firm adhesion. Among these, VCAM-1 seems to play a crucial role, as immunoblockade of this adhesion molecule has the highest impact on leukocyte adhesion in TNBS-induced colitis [14] and is more potent than ICAM-1 or MAdCAM-1 immunoblockade in reducing inflammatory lesions in experimental colitis [15]. Previous in vitro studies using antioxidants support the idea that ROS are obligate intermediates in endothelial cell adhesion molecule induction, and these treatments also significantly block leukocyte infiltration into tissues, which depend on the expression of these molecules [43, 44]. Two recent in vivo studies using SOD gene transfer demonstrate that SOD overexpression results in a reduced VCAM-1 up-regulation in response to irradiation [34] or hypertension [45] by decreasing superoxide levels. Paralleling these observations, in the current study, we demonstrate that treatment with the highest dose of SOD markedly attenuated VCAM-1 expression in the colon of colitic rats, and this was followed by a significant reduction in the number of firmly adherent leukocytes and amelioration of colitis. The potential effect of SOD treatment preventing ICAM-1 up-regulation in TNBS colitis, demonstrated by Cuzzocrea et al. [10], may also contribute to limit leukocyte recruitment, as immunoneutralization of this adhesion molecule also reduces firm leukocyte adhesion in colonic venules, although to a lesser extent than VCAM-1 immunoneutralization [14]. Taken together, these observations indicate that modulation of the expression of endothelial adhesion molecules of the Ig superfamily is important in regulating vascular inflammatory processes in response to treatment with antioxidants such as SOD.

Reduction in oxidant stress in the course of intestinal inflammation may have effects, which contribute to the beneficial action observed in the TNBS model of colitis, on many components of the inflammatory cascade, in addition to adhesion molecule expression. In that regard, a decreased production of the highly cytotoxic oxidant peroxynitrite, which results from the interaction between nitric oxide and superoxide in the inflamed colon [46], may be one of the factors contributing to the beneficial effects of SOD in intestinal inflammation.

In conclusion, the protective effects of SOD demonstrated in this study shed new light on our understanding of the role of oxidative stress, adhesion molecule expression, and leukocyte recruitment in the pathogenesis of colonic inflammation. Our data suggest that SOD treatment decreases ROS generation and oxidative stress and thus, inhibits endothelial activation and indicate that modulation of factors that govern adhesion molecule expression and leukocyte-endothelial interactions, such as antioxidants, may be important, new tools for the treatment of IBD.

## ACKNOWLEDGMENTS

This work was supported by Grant SAF2002-02211 from Ministerio de Ciencia y Tecnología, and Grant C03/02 from Instituto de Salud Carlos III supported this work. J. S. is a recipient of a grant from Ministerio de Ciencia y Tecnología.

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