Erythropoietin Reduces the Development of Experimental Inflammatory Bowel Disease

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Received June 24, 2004; accepted August 6, 2004

ABSTRACT
Inflammatory bowel disease is characterized by oxidative and nitrosative stress, leukocyte infiltration, and up-regulation of the expression of intercellular adhesion molecule-1 (ICAM-1) in the colon. Erythropoietin (EPO) is a potent stimulator of erythroid progenitor cells, and its expression is enhanced by hypoxia. Here we investigate the effects EPO has on the development of experimental colitis. To address this question, we used an experimental model of colitis induced by dinitrobenzene sulfonic acid (DNBS). When compared with DNBS-treated mice, EPO (1000 IU/kg day s.c.)-treated mice subjected to DNBS-induced colitis experienced significantly lower rates in the extent and severity of the histological signs of colon injury. DNBS-treated mice experienced diarrhea and weight loss. At 4 days after administration of DNBS, the mucosa of the colon exhibited large areas of necrosis. Neutrophil infiltration (determined by histology as well as an increase in myeloperoxidase activity in the mucosa) was associated with up-regulation of ICAM-1. Immunohistochemistry for nitrotyrosine and poly(ADP-ribose) showed an intense staining in the inflamed colon. On the contrary, the treatment of DNBS-treated mice with EPO significantly reduced the degree of diarrhea and weight loss caused by administration of DNBS. EPO also caused a substantial reduction of the degree of colon injury, the rise in myeloperoxidase activity (mucosa), and the increase in staining (immunohistochemistry) for nitrotyrosine as well as the up-regulation of ICAM-1 caused by DNBS in the colon. Thus, treatment of rats with EPO reduces the degree of colitis caused by DNBS. We propose that EPO may be useful in the treatment of inflammatory bowel disease.

The inflammatory bowel diseases (IBD), which include Crohn’s disease and ulcerative colitis, have become important health problems in recent years. With an actual prevalence of 200 to 500 per 100,000 people in Western countries and an incidence of about 20 per every 100,000 people, the prevalence in high-incidence areas almost doubles every 10 years. Indeed, both are chronic diseases that affect people of a relatively young age. In the last decade, there has been a shift toward an increase in the incidence of Crohn’s disease in areas with a high incidence of IBD, and IBD has tended to occur at all ages (Podolsky, 2002). Substantial progress has been made in characterizing immune-cell populations and inflammatory mediators in patients with inflammatory bowel disease and in murine models. It is well accepted that the mucosa of patients with established Crohn’s disease is dominated by CD4+ lymphocytes with a type 1 helper-T-cell phenotype, characterized by the production of interferon-γ, interleukin-2, and tumor necrosis factor-α (TNF-α) (Papadakis and Targan, 2000), and activation of nuclear factor κB (NF-κB) (Barnes and Karin, 1997). In contrast, the mucosa in patients with ulcerative colitis may be dominated by CD4+ lymphocytes with an atypical type 2 helper-T-cell phenotype, characterized by the production of transforming growth factor-β and interleukin-5 but not interleukin-4 (Fiocchi, 1998). In addition, macrophages play a main role in the formation of noncaseous epithelioid granuloma in the intestinal mucosa, which is characteristic of Crohn’s disease, and are also involved in the mucosal immune response. Activated macrophages produce cytokines such as TNF-α, interleukin (IL)-6, IL-8, and others. The biological action of TNF-α and...
IL-6 is the main factor in the pathogenesis of Crohn’s disease, and the regulation of this process is very important in controlling the disease (Sandborn and Hanauer, 1999). In addition, recruitment of inflammatory cells from circulation is an important process in augmenting inflammatory response. TNF-α and IL-6 induce the expression of adhesion molecules in the vascular endothelium, and invasion of inflammatory cells into mucosal layer subsequently occurs. Selectins, intercellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule-1 (VCAM-1), which are expressed at the surface of vascular endothelium, are involved in this process (Koizumi et al., 1992). Moreover, it is well known that superoxide radical production and granular release are induced by the recruited leukocytes, especially granulocytes, and non-specific inflammatory substances are subsequently produced. The main substances are arachidonic acid metabolites (such as thromboxane A₂, leukotriene B₄, and prostaglandin E₂), reactive oxygen metabolites, free radicals, and nitric oxide. Reactive oxygen metabolites and nitric oxide play an important role in direct injury against the intestinal mucosa (Grisham, 1994; Cuzzocrea et al., 2001). Novel approaches that protect and salvage injured colon tissue would constitute important advances in the therapy of IBD.

Erythropoietin (EPO) is a glycoprotein hormone produced primarily by the adult kidney in the regulation of red blood cell production, exerting its hematopoietic effects by stimulating the proliferation of committed erythroid progenitor cells and their development into mature erythrocytes (Jelkmann, 1992). In its originally recognized (classical) role, EPO and its receptor (EPOR) are indispensable for the survival, proliferation, and differentiation of erythroid progenitor cells. From a broad perspective, EPO and EPOR function as the primary mediators of a general protective response to tissue hypoxia by acting to maintain adequate tissue oxygenation through adjustments of circulating red-cell mass by using a hormonal feedback control system involving the kidney and the bone marrow. More recently, it has been recognized that EPO and EPORs are also expressed by other tissues and organs, including the brain and heart (Masuda et al., 1999). The critical importance of these proteins is proven by knockout experiments targeting either the EPO or EPOR genes. Either genotype is embryonically lethal because erythrocyte apoptosis leads to major developmental abnormalities of the central nervous system (Yu et al., 2002) and the heart (Wu et al., 1999). EPO has also been shown to stimulate mitosis and signaling in astrocytes (Sugawa et al., 2002), endothelial cells (Jaquet et al., 2002), cardiomyoblasts (Ogilvie et al., 2000), and cardiomyocytes (Wald et al., 1996) maintained in vitro.

Therefore, there are several recent reports documenting that EPO reduces the injury caused by ischemia/reperfusion of the brain (Agnello et al., 2002), eye (Junk et al., 2002), gut (Squadrito et al., 1999), heart (Parsa et al., 2003), and kidney (Sharples et al., 2003).

Encouraged by the substantial body of evidence demonstrating the sizable beneficial effects of EPO in models of nervous system injury and ischemia and reperfusion, the present study was designed to evaluate the possible beneficial effects of EPO treatment in rodent models of dinitrobenzene sulfonic acid (DNBS)-induced colitis. To gain a better insight into the mechanism(s) of action of the observed anti-inflammatory effects of EPO, we have also investigated the effects of EPO on the degree of colonic injury, the rise in myeloperoxidase (MPO) activity (mucosa), the production of TNF-α and IL-1β (colon levels), the increase in staining (immunohistochemistry) for nitrotyrosine, and the increased expression of ICAM-1 caused by DNBS in the colon.

**Materials and Methods**

**Animals.** The study was carried out in 6- to 8-week-old CD1 male mice (Charles River Breeding Laboratories, Calco, Italy). The animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the European Economic Community regulations (O.J. of E.C. L 358/1 12/18/1986).

**Experimental Groups.** EPO was given daily as an s.c. bolus injection (1000 IU/kg, DNBS + EPO group) starting from day 2. In a vehicle-treated group of mice, vehicle (saline) was given instead of EPO (DNBS group). In a separate group of mice, surgery was performed with every aspect identical to the one in the DNBS group except that saline was injected instead of DNBS (Sham group). In an additional group of animals, sham-surgery was combined with the administration of EPO (dose as above) (Sham + EPO group).

**Induction of Experimental Colitis.** Colitis was induced with a very low dose of DNBS (4 mg per mouse) by using a modification (Morris et al., 1989) of the method first described in rats (Wallace et al., 1995). In preliminary experiments, this dose of DNBS was found to induce reproducible colitis without mortality. Mice were anesthetized by enflurane. DNBS (4 mg in 100 μl of 50% ethanol) was injected into the rectum through a catheter inserted 4.5 cm proximally to the anus. Carrier alone (100 μl of 50% ethanol) was administered in control experiments. Thereafter, the animals were kept for 15 min in a Trendelenburg position to avoid reflux. After colitis and sham-colitis induction, the animals were observed for 3 days. On day 4, the animals were weighed and anesthetized with chloral hydrate, and the abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border, rinsed, weighed, and processed for histology and immunohistochemistry. Colon damage (macroscopic damage score) was evaluated and scored by two independent observers as described previously (Wallace and Keenan, 1990) according to the following criteria: 0, no damage; 1, localized hyperemia without ulcers; 2, linear ulcers with no significant inflammation; 3, linear ulcers with inflammation at one site; 4, two or more major sites of inflammation and ulceration extending >1 cm along the length of the colon; and 5, through 8, one point being added for each centimeter of ulceration beyond an initial 1 cm.

**Light Microscopy.** After fixation for 1 week at room temperature in Dietrich solution (14.25% ethanol, 1.85% formaldehyde, and 1% acetic acid), samples were dehydrated in graded ethanol and embedded in paraplast (Sherwood Medical, St. Louis, MO). Thereafter, 7-μm sections were deparaffinized with xylene, stained with hematoxylin-eosin and trichromic von Giesson’s stain, and observed in a DiLux 22 Leitz microscope (Leitz, Wetzlar, Germany). To have a quantitative estimation of colon damage, each section (n = 6 for each animal) was scored by two independent observers blinded to the experimental protocol. The following morphological criteria were considered: score 0, no damage; score 1 (mild), focal epithelial edema and necrosis; score 2 (moderate), diffuse swelling and necrosis of the villi; score 3 (severe), necrosis with presence of neutrophil infiltrate in the submucosa; and score 4 (highly severe), widespread necrosis with massive neutrophil infiltrate and hemorrhage.

**Myeloperoxidase Activity.** MPO activity, an indicator of polymorphonuclear leukocyte accumulation, was determined as previously described (Cuzzocrea et al., 2001). Four days after intracolic injection of DNBS, the colon was removed and weighed. The colon...
was homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 37°C and was expressed in milliunits per gram weight of wet tissue.

Measurement of Cytokines. Portions of terminal colon, collected 4 days after intracolonic injection of DNBS, were homogenized as previously described (Diaz-Granados et al., 2000) in PBS containing 2 mM of phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO). The levels of TNF-α and IL-1β were evaluated in the colon 4 days after intracolonic injection of DNBS. These parameters were measured by using a commercial colorimetric kit (Calbiochem-Novabiochem, San Diego, CA).

Localization of Nitrotyrosine, PAR, and ICAM-1 by Immunohistochemistry. At the end of the experiment, the tissues were fixed in 10% PBS-buffered formaldehyde, and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). Sections were incubated overnight with anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS) or with anti-ICAM-1 polyclonal antibody (CD54) (1:500 in PBS, v/v) or with anti-PAR (1:500 in PBS, v/v) (DBA). Specific labeling was detected with a biotin-conjugated goat anti-rabbit, donkey anti-goat, or goat anti-mouse IgG and avidin-biotin peroxidase complex (DBA). To verify the binding specificity for ICAM-1 and PAR, some sections were also incubated with primary antibody only (no secondary antibody) or with secondary antibody only (no primary antibody). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all the experiments. To confirm that the immunoreactions for the nitrotyrosine were specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. Immunocytochemistry photographs (n = 5) were assessed by densitometry by using Optilab Graftek software on a Macintosh personal computer.

Reagents. Recombinant human EPO was obtained from St. Bartholomew’s Hospital Pharmacy (London, UK). Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG, and avidin-biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, CA). Primary anti-nitrotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Primary ICAM-1 (CD54) for immunohistochemistry was purchased by BD PharMingen (San Diego, CA). Reagents and secondary and nonspecific IgG antibody for immunohistochemical analysis were from Vector Laboratories. All other reagents and compounds used were obtained from Sigma-Aldrich.

Statistical Analysis. All values in the figures and text are expressed as mean ± S.E.M. of N observations, where n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days.
The results were analyzed by one- and two-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. Nonparametric data were analyzed with Fisher’s exact test. A P value of less than 0.05 was considered significant.

**Results**

**Effects of EPO Treatment on the Degree of Colitis (Histology and General Assessment).** Four days after intracolonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. The macroscopic inspection of cecum, colon, and rectum showed presence of mucosal congestion, erosion, and hemorrhagic ulcerations (see damage score, Fig. 1A). The histopathological features included a trans-mural necrosis and edema and a diffuse leukocyte cellular infiltrate as well as lymphocyte in the submucosa of colon section from DNBS-treated mice (Figs. 1B and 2B). The observed inflammatory changes of the large intestine were associated with an increase in the weight of the colon (Fig. 1C). Four days after colitis induced by DNBS treatment, all mice had diarrhea and a significant reduction in body weight (compared with the control groups of mice) (Fig. 1D). Daily treatment with EPO (1000 IU/kg) resulted in a significant decrease in the extent and severity of damage (Figs. 1 and 2C) as well as a significant reduction of the lymphocyte infiltration. No histological alteration was observed in the colon tissue from vehicle-treated mice (Figs. 1 and 2B).

**Effects of EPO Treatment on Production of TNF-α and IL-1β after DNBS Administration.** To test whether...
EPO treatment may modulate the inflammatory process through the regulation of the secretion of others cytokines, we analyzed the colon levels of pro-inflammatory cytokines TNF-\(\alpha\) and IL-1\(\beta\) in mice treated with EPO. A substantial increase of TNF-\(\alpha\) and IL-1\(\beta\) formation was found in colon samples collected from DNBS-treated mice 4 days after DNBS administration (Fig. 3). Colon levels of TNF-\(\alpha\) and IL-1\(\beta\) were significantly reduced in the colon tissues collected from DNBS-treated mice after administration of EPO (Fig. 3).

**Effects of EPO Treatment on ICAM-1 Expression and Polymorphonuclear Neutrophil (PMN) Infiltration.** The colitis caused by DNBS was also characterized by an increase in myeloperoxidase activity, an indicator of the accumulation of PMNs in the colon (Fig. 4). This finding is consistent with the observation made with light microscopy that the colon of vehicle-treated DNBS rats contained a large number of PMNs. EPO treatment significantly reduced the degree of PMN infiltration (determined as increase in MPO activity) in inflamed colon (Fig. 4). To further elucidate the effect of EPO on PMN accumulation in inflamed colon, we evaluated the intestinal expression of ICAM-1. Tissue sections obtained from sham-operated mice with anti-ICAM-1 antibody showed a specific staining along the vessels, demonstrating that ICAM-1 is expressed constitutively in endothelial cells (Fig. 5). After DNBS administration, the staining intensity substantially increased in the vessels of the lamina propria and submucosa. Immunohistochemical staining for ICAM-1 was also present in epithelial cells of injured colon and in infiltrated inflammatory cells in damaged tissues from DNBS-treated wild-type mice (Figs. 5 and 6B). Sections from EPO-treated mice did not reveal any up-regulation of the constitutive ICAM-1, which was normally expressed in the endothelium along the vascular wall (Figs. 5 and 6C).

**Effects of EPO Treatment on Nitrotyrosine and PAR Formation.** To determine the localization of “peroxynitrite formation” and/or other nitrogen derivatives produced during colitis, nitrotyrosine, a specific marker of nitrosative stress, was measured by immunohistochemical analysis in the distal colon. Sections of colon from sham-administered mice did not stain for nitrotyrosine (Fig. 5). Colon sections obtained from vehicle-treated DNBS-treated mice exhibited positive staining for nitrotyrosine (Figs. 5 and 7B) and for poly(ADP-ribose) (Figs. 5 and 8B) localized in inflammatory cells and in disrupted epithelial cells. Sections from EPO-treated mice did not reveal any positive staining for nitrotyrosine (Figs. 5 and 7C) and PAR (Figs. 5 and 8C). No positive staining for either nitrotyrosine or PAR was found in the colon section from sham-treated mice (Figs. 7A and 8A).

**Discussion**

Erythropoietin, the critical hormone promoting the survival and differentiation of erythroid progenitor cells, is currently being used in the therapy of patients with chronic renal failure suffering from anemia (Cody et al., 2001). Here we demonstrate for the first time that EPO also reduces the development of experimental colitis. Specifically, we demonstrate that treatment with EPO significantly reduced the degree of diarrhea and weight loss, the degree of colonic injury, the infiltration of the colon PMNs, the positive staining (immunohistochemistry) for nitrotyrosine and PAR, and the increased expression of ICAM-1 caused by DNBS in the colon. All of these findings support the view that EPO exerts potent anti-inflammatory effects. What, then, is the mechanism by which EPO inhibits colon inflammation caused by injection of DNBS?

**Effect of EPO on Oxidative and Nitrosative Damage in DNBS-Induced Colitis.** IBD is a multifactorial disorder of unknown etiology. There is, however, very good evidence from animal and clinical studies that documents that an enhanced formation of reactive oxygen or nitrogen species importantly contributes to the pathophysiology of IBD. For instance, monocytes from patients with Crohn’s disease (Kita-hora et al., 1988) and PMNs from patients with ulcerative colitis (Shiratora et al., 1993) have an increased capacity to generate free oxygen radicals. Furthermore, advanced stages of bowel inflammation in humans (Boughton-Smith et al., 1993; Middleton et al., 1993) and animals (Ai ko and Grisham, 1995; Cuzzocrea et al., 2001) are associated with an
enhanced (local) formation of NO by inducible NO synthase. In the present study, we confirm that the mucosal damage induced by intracolonic administration of DNBS was associated with immunohistochemical expression of nitrotyrosine, mostly localized on epithelial cells and in the area of infiltrated inflammatory cells; this suggests that peroxynitrite or other nitrogen derivatives and oxidants are formed in vivo and may contribute to tissue injury. These data are consistent with previous findings that immunohistochemical staining for nitrotyrosine was localized on epithelial cells in a DNBS model of guinea pig ileitis (Miller et al., 1995) or rat colitis (Zingarelli et al., 1999b; Cuzzocrea et al., 2001) and in active Crohn’s lesions in humans (Singer et al., 1996). The pathogenic role of nitrogen-derived species such as peroxynitrite in inflammatory bowel disease is further supported by the fact that intracolonic administration of exogenous peroxynitrite induces a severe colonic inflammation that mimics the features of both ulcerative colitis and Crohn’s disease (Rachmilewitz et al., 1993). In the present study, we observed that epithelial disruption was significantly less in mice treated with EPO. Indeed, EPO treatment prevented the formation of tissue nitrotyrosine staining in DNBS-treated animals. This result of the effects of EPO on free radical production is in agreement with previous studies (Squadrito et al., 1999; Hirayama et al., 2002). Superoxide and peroxynitrite cause DNA single-strand damage, leading to poly(ADP-ribose) synthetase activation and cell death (Szabo and Dawson, 1998). Some evidence exists to support the possible role of poly(ADP-ribose) synthetase activation in inflammatory bowel disease (Zingarelli et al., 1999a; Mazzon et al., 2002). As shown in Fig. 8, EPO reduced poly(ADP-ribose) synthetase immunofluorescence, an effect that might account for the overall protective action of EPO.

The Beneficial Effect of EPO in DNBS-Induced Colitis Is Related to an Inhibition of Cytokine Production. TNF-α and IL-1β are clearly involved in the pathogenesis of colitis since these cytokines are present in colon tissue and can be detected immunohistochemically in the inflamed tissues (Carty et al., 2000). Direct evidence that TNF-α and IL-1β play a role in the pathogenesis of experimental colitis has been obtained in animal models in which blocking of the action of these cytokines has been shown to delay the onset of experimental colitis, suppress inflammation, and ameliorate colon destruction that corresponds to the anti-inflammatory response (Sandborn and Hanauer, 1999). In such cases, significant reduction in Crohn’s disease activity index as well as attenuation of attenuated histopathologic and endoscopic inflammation in Crohn’s disease patients was observed. We confirm that the model of colitis used here leads to a substantial increase in the levels of TNF-α and IL-1β in the colon. Interestingly, the levels of these two pro-inflammatory cytokines are significantly lower in the DNBS-treated mice that were treated with EPO. These findings, therefore, suggest that EPO reduced the activation and the subsequent expression of pro-inflammatory genes.
The Beneficial Effect of EPO in DNBS-Induced Colitis Is Related to an Alteration in Neutrophil Recruitment. Neutrophils play a crucial role in the development and full manifestation of gastrointestinal inflammation because they represent a major source of free radicals in the inflamed colonic mucosa (Grisham, 1994; Cuzzocrea et al., 2001). Neutrophil infiltration into inflamed tissue plays a crucial role in the destruction of foreign antigens and in the breakdown and remodeling of injured tissue. The interactions of polymorphonuclear cells with the endothelium are regulated by various adhesion molecules, including the selectins, the $\beta_2$ integrins, and adhesion molecules of the immunoglobulin superfamily (Geng et al., 1990). Although P-selectin is necessary for early contact of neutrophil with the endothelium, a P-selectin-mediated leukocyte-endothelial interaction is not sufficient to allow neutrophil emigration from the vessel. A more firm adherence of the neutrophil to the endothelial surface is required for transendothelial migration. This firm adherence involves the interaction of $\beta_2$ integrins (i.e., CD11/CD18) on the polymorphonuclear cells surface and ICAM-1 on the endothelial cell surface (Koizumi et al., 1992). A major finding of this study was that not only did the EPO-treated mice show a remarkable recovery of the mucosal morphology associated with a reduction in oxidative and nitrosative damage after DNBS administration but that in EPO-treated mice, infiltration of polymorphonuclear neutrophils was significantly reduced in tissue. Furthermore, ICAM-1 was expressed in endothelial and epithelial cells and neutrophils in the distal colon in DNBS-treated mice. This was associated with a significant reduction of ICAM-1 expression in endothelial and epithelial cells. Our data suggest that EPO treatment reduced the neutrophil infiltration and is consistent with published previously data (Rey-Ladino et al., 1999; Makis et al., 2001).

Summary and Conclusions. Our results demonstrate that EPO is protective in experimental colitis and that inhibition of TNF-α formation (among other myeloperoxidase effects that include inhibition of neutrophils infiltration) in the colon probably accounts for its beneficial effects. Future studies are needed to clarify the exact mechanisms beside the anti-inflammatory property of EPO treatment; however, there are several potential explanations for this finding. In fact, it has been demonstrated that treatment of mice with EPO for 3 days may well have resulted in the up-regulation of protective genes, including endothelial NO synthase, manganese superoxide dismutase, and 70-kDa heat shock protein (HSP70), which in turn could have contributed to the observed protective effect.

Based on the findings above and with full recognition that DNBS-induced colitis does not mimic the clinical disease in its full and complicated etiology, it is not unreasonable to propose that EPO may be valuable as a clinical candidate for such disorders.
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Fig. 8. Immunohistochemical localization of PAR in the colon. Immunohistochemical analysis for PAR (B) shows positive staining localized in the injured area from DNBS-treated mice. The intensity of the positive staining for PAR (C) was markedly reduced in the tissue section obtained from sham-treated mice. Figure is representative of at least three experiments performed on different experimental days.

Acknowledgments

We thank Giovanni Pergolizzi and Carmelo La Spada for excellent technical assistance during this study, Caterina Cutrona for secretarial assistance, and Valentina Malvagni for editorial assistance with the manuscript.

References


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