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Tempol, a membrane-permeable radical scavenger, reduces dinitrobenzene sulfonic acid-induced colitis

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Abstract

Inflammatory bowel disease is characterized by oxidative and nitrosative stress, leukocyte infiltration, and up-regulation of intercellular adhesion molecule 1 (ICAM-1) expression in the colon. The aim of the present study was to examine the effects of tempol, a membrane-permeable radical scavenger, in rats subjected to experimental colitis. Colitis was induced in rats by intracolonic instillation of dinitrobenzene sulfonic acid. Rats experienced bloody diarrhea and significant loss of body weight. At 4 days after the administration of dinitrobenzene sulfonic acid, the colon injury comprised of large areas of mucosal necrosis. Neutrophil infiltration (measured as increase in myeloperoxidase activity in the mucosa) was associated with up-regulation of ICAM-1 and expression of P-selectin and high levels of malondialdehyde (an indicator of lipid peroxidation). Immunohistochemistry for nitrotyrosine and poly (ADP-ribose) synthetase showed an intense staining in the inflamed colon. Treatment of rats with tempol (15 mg/kg daily i.p.) significantly reduced the appearance of diarrhea and the loss in body weight. This was associated with a remarkable amelioration of the disruption of the colonic architecture as well as a significant reduction in the degree of both neutrophil infiltration and lipid peroxidation in the inflamed colon. Tempol also reduced the appearance of nitrotyrosine and poly (ADP-ribose) synthetase immunoreactivity in the colon as well as the up-regulation of ICAM-1 and P-selectin. The results of this study suggest that membrane-permeable radical scavengers, such as tempol, exert beneficial effects in experimental colitis and may, hence, be useful in the treatment of inflammatory bowel disease.

Keywords: Dinitrobenzene sulfonic acid; Inflammation; Nitric oxide (NO); Peroxynitrite; Poly (ADP-ribose) synthetase; Colon damage; Free radical

1. Introduction

Reactive oxygen and nitrogen species have been implicated as mediators of the disruption of the intestinal barrier in inflammatory bowel diseases (Rachmilewitz et al., 1993; Middleton et al., 1993; Grisham, 1994). In addition to reactive oxygen, an overproduction of nitric oxide (NO) due to the expression of the inducible isoform of NO synthase (iNOS) also plays important role in various models of inflammation including colitis (Nathan, 1996; Cuzzocrea et al., 1998a). Patients with Crohn's disease or ulcerative colitis exhibit elevated levels of nitrite (metabolite of NO in water) in rectal dialysates and increased iNOS activity in colonic biopsies (Middleton et al., 1993; Ikeda et al., 1997). Furthermore, inhibitors of iNOS activity (or induction) exert beneficial effects in animal models of experimental colitis (Aiko and Grisham, 1995; Mourelle et al., 1996). It has been suggested that the damaging effects of NO are mediated, at least in part, by peroxynitrite ($ONOO^-$), a potent oxidant produced by the reaction

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of NO with superoxide anions (Beckman, 1996). $ONOO^-$ is cytotoxic via a number of independent mechanisms including (i) the initiation of lipid peroxidation, (ii) the inactivation of the activity of a variety of enzymes (most notably, mitochondrial respiratory enzymes and membrane pumps) (Crow and Beckman, 1995), and (iii) depletion of glutathione (Phelps et al., 1995). Moreover, $ONOO^-$ can also cause DNA damage (Salgo et al., 1995) resulting in the activation of the nuclear enzyme poly (ADP-ribose) synthetase, depletion of NAD and ATP and ultimately cell death (Szabó et al., 1997). The activation of poly (ADP-ribose) synthetase may also play an important role in various models of inflammation including colitis (Zingarelli et al., 1999a). In this condition, pharmacological inhibition or gene targeted disruption of poly (ADP-ribose) synthetase appears to exert beneficial effects (Szabó et al., 1997, 1998; Cuzzocrea et al., 1998b,c; Zingarelli et al., 1999a).

Interventions, which reduce the generation or the effects of reactive oxygen, exert beneficial effects in a variety of models of inflammation including the dinitrobenzene sulfonic acid-induced colitis model used here. These therapeutic interventions include melatonin (Cuzzocrea et al., 2000), a vitamin E-like antioxidant (Cuzzocrea et al., 1999a), a superoxide dismutase-mimetic (Cuzzocrea et al., 1999b) and a peroxynitrite decomposition catalyst (Salvemini et al., 1998). The therapeutic efficacy of superoxide dismutase itself in animals with systemic inflammation, haemorrhage and shock is controversial. The following reasons may explain the lack of effect of superoxide dismutase against the tissue injury associated with local or systemic inflammation. (1) Superoxide dismutase scavenges superoxide, but without efficient removal of the hydrogen peroxide which is produced, levels of hydroxyl radicals may increase (Goode and Webster, 1993). Indeed, superoxide dismutase may function as a pro-oxidant by catalysing the conversion of hydrogen peroxide to hydroxyl radicals (Yim et al., 1990). (2) Neither superoxide dismutase nor superoxide anions easily cross biological membranes. Thus, an increase in the amounts of extracellular superoxide dismutase does not attenuate the effects of superoxide anions generated by intracellular sources (Fridovich, 1995). In contrast to superoxide dismutase, spin trapping nitrones, such as phenyl *N*-*tert*-butyl nitrone, consistently improve the outcome in rat models of endotoxic (McKechnie et al., 1986; Hamburger and Mccay, 1989) and traumatic shock (Novelli et al., 1986; Novelli, 1992). Similarly, tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), a stable piperidine nitroxide (stable free radical) of low molecular weight, which permeates biological membranes and scavenges superoxide anions in vitro (Laight et al., 1997a,b), also exerts beneficial effects in rats subjected to endotoxaemia (Leach et al., 1998).

Here, we investigate the effects of tempol on the inflammatory response (colitis) caused by intracolonic administration of dinitrobenzene sulfonic acid.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (300–350 g, Charles River, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

2.2. Experimental groups

In the treated group of animals, tempol was given daily as an i.p. bolus injection (15 mg/kg every 24 h) starting from day 1. (dinitrobenzene sulfonic acid + tempol group; $n = 15$). In a vehicle-treated group of rats, vehicle (saline) was given instead of tempol (dinitrobenzene sulfonic acid group; $n = 15$). In separate groups of rats, surgery was performed in its every aspect identical to the one in the dinitrobenzene sulfonic acid group, except that 50% ethanol (vehicle for dinitrobenzene sulfonic acid) was injected instead of dinitrobenzene sulfonic acid (sham group; $n = 15$). An additional group of sham-operated animals were treated with tempol (dose as above) (Sham + tempol; $n = 15$).

2.3. Induction of experimental colitis

Colitis was induced by using a technique of acid-induced colonic inflammation as described previously (Fries et al., 1999). Fasted rats were lightly anaesthetized with isoflurane. Then, a 3.5 F catheter was inserted into the colon via the anus until approximately, the splenic flexure (8 cm from the anus). The 2,4,6-dinitrobenzene sulfonic acid (25 mg/rat) was dissolved in 50% ethanol (total volume, 0.8 ml) and deposited in the colon. Thereafter, the animals were kept for 15 min in a Trendelenburg position to avoid reflux. After administration of dinitrobenzene sulfonic acid or its vehicle, the animals were observed for 3 days. On day 4, the animals were weighed and anaesthetised with chloralium hydrate (400 mg/kg, i.p.), and the abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues, opened along the anti-mesenteric border, rinsed, weighed and processed for histology and immunohistochemistry. In an additional experiment, animals (10 animals/group) were monitored for 7 days in order to evaluate the effect of dinitrobenzene sulfonic acid in the presence or absence of tempol on mortality.

2.4. Optical microscopy

After fixation for 1 week at room temperature in Dietrich solution (14.25% ethanol, 1.85% formaldehyde, 1%

acetic acid), samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, New Jersey). Thereafter, 7 μm sections were deparaffinized with xylene, stained with hematoxylin-eosin and trichromic van Gieson's stain, and examined in a Dialux 22 Leitz (Wetziar, Germany) microscope. The macroscopic damage score, according to Wallace et al. (1992), was assessed by an investigator blinded for the treatment regime.

2.5. Immunofluorescence localisation for nitrotyrosine, poly (ADP-ribose) synthetase, P-Selectin and for intercellular adhesion molecule 1 (ICAM-I)

Indirect immunofluorescence staining was performed on 7 μm thick sections of unfixed rat colon. Sections were cut with a Slee and London cryostat at −30°C, transferred into clean glass slides and dried overnight at room temperature. Sections were permeabilized with acetone at −20°C for 10 min and re-hydrated in phosphate buffered saline (150 mM NaCI, 20 MM sodium phosphate pH 7.2) at room temperature for 45 min. Sections were co-incubated overnight: (1) with anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v) or with anti-poly (ADP-Ribose) goat polyclonal antibody rat (1:500 in phosphate buffered saline, v/v); (2) with rabbit anti-human polyclonal antibody directed at P-Selectin (CD62P), which reacts with rat and with mouse anti-rat antibody directed at ICAM-1 (CD54) (1:500 in phosphate buffered saline, v/v) (DBA, Milan, Italy). Sections were washed with phosphate buffered saline, and co-incubated with secondary antibody (tetramethyl rhodamine-conjugated anti-rabbit and with Fluorescein isothiocyanate-conjugated anti-mouse (Jackson, West Grove, PA) or with tetramethyl rhodamine-conjugated anti-goat antibody (1:80 in phosphate buffered saline, v/v) for 2 h at room temperature. Sections were washed as before, mounted with 90% glycerol in phosphate buffered saline, and observed with a Nikon RCM8000 confocal microscope equipped with a 40× oil objective.

2.6. Myeloperoxidase activity

Myeloperoxidase activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (Mullane et al., 1985). At the specified time following the intracolonic injection of dinitrobenzene sulfonic acid, colon was obtained and weighed. The tissue was homogenised in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 × *g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min^{-1} at 37°C and was expressed in milliunits per gram weight of wet tissue.

2.7. Malondialdehyde measurement

Malondialdehyde levels in the colon were determined as an indicator of lipid peroxidation (Ohkawa et al., 1979). The tissue was homogenized in 1.15% KCl solution. An aliquot (100 μl) of the homogenate was then added to a reaction mixture containing 200 μl of 8.1% sodium dodecyl sulfate (SDS), 1500 μl of 20% acetic acid (pH 3.5), 1500 μl of 0.8% thiobarbituric acid and 700 μl distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

2.8. Data analysis

All values in the figures and text are expressed as mean ± standard error (S.E.) of the mean of n observations. Statistical analysis for survival data was calculated by Fisher's exact probability test. For the in vivo studies, *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 analysed by one-way analysis of variance followed by a Bonferroni post-hoc test for multiple comparisons. A *P*-value of less than 0.05 was considered significant.

2.9. Reagents

Primary anti-nitrotyrosine antibody was purchased from Upstate Biotech (Saranac Lake, NY). Primary P-selectin

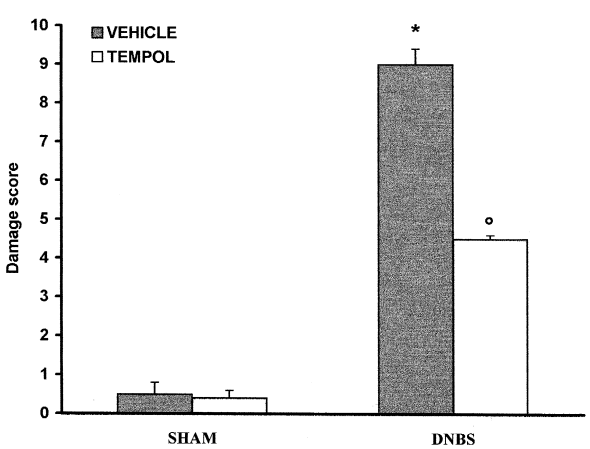

Fig. 1. Effect of tempol treatment on the damage score. Colonic damage was scored on a scale of 0 (normal)–10 (severe) by two independent observers. Values are means ± S.E. of 15 rats for each group. * $P < 0.01$ vs. sham; ° $P < 0.01$ vs. dinitrobenzene sulfonic acid.

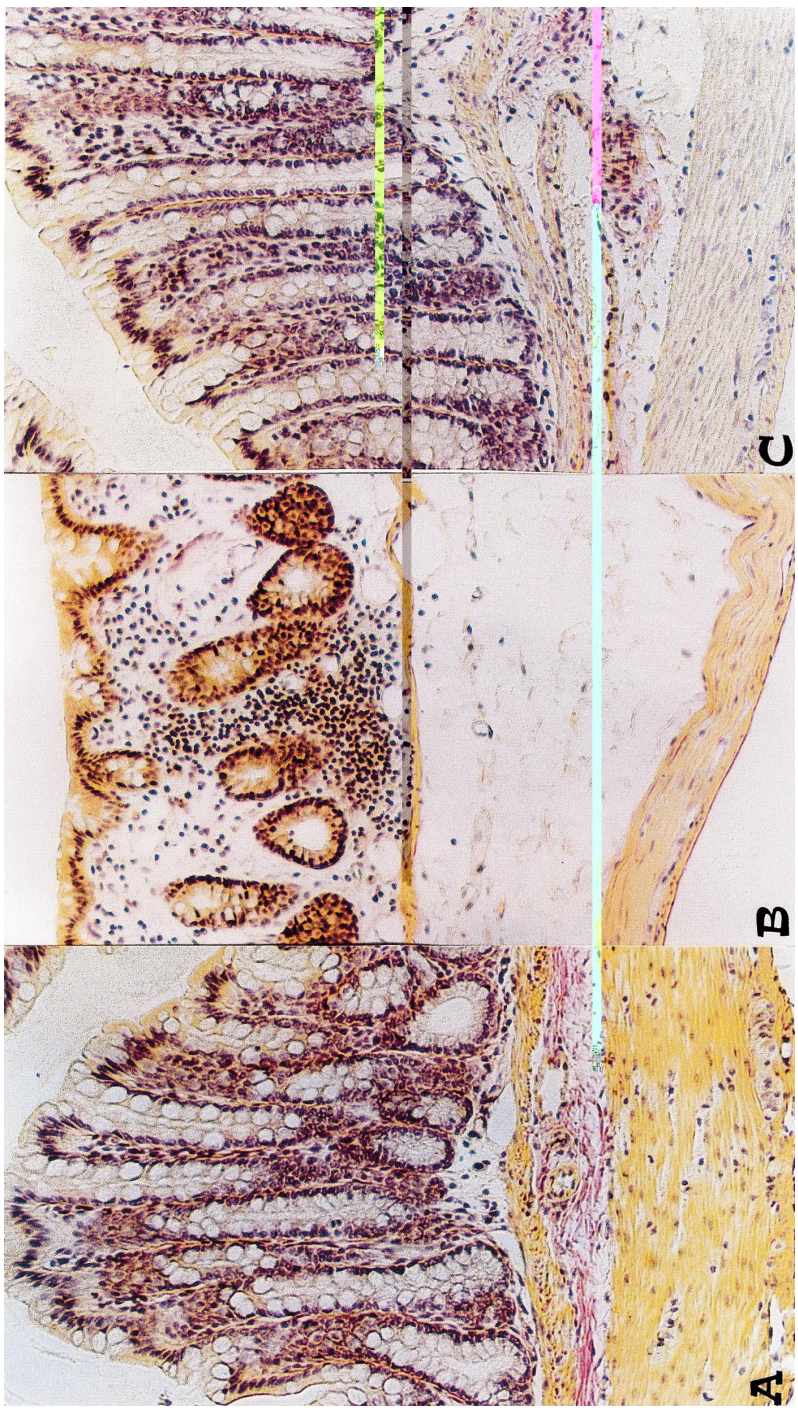

Fig. 2. Effect of tempol on colon injury. No histological modification was observed in mucosal from sham-operated rats (A). Mucosal injury was produced after dinitrobenzene sulfonic acid administration characterized by absence of epithelium and a massive mucosal and submucosal infiltration of inflammatory cells (B). Treatment with tempol (C) corrected the disturbances in morphology associated with dinitrobenzene sulfonic acid administration. Original magnification: ×63. Figure is representative of at least three experiments performed on different experimental days.

(CD62P) and ICAM-1 (CD54) were purchased from Pharmingen (DBA, Milan, Italy). All other reagents and compounds used were purchased from Sigma (Sigma, St. Louis, MO).

3. Results

Four days after intracolonic administration of dinitrobenzene sulfonic acid, the colon appeared flaccid and filled with liquid. The cecum, colon and rectum showed evidence of mucosal congestion, erosions and hemorrhagic ulcerations (see Fig. 1 for damage score). The histopathological features included a transmural necrosis and edema and a diffuse leukocyte cellular infiltrate in the submucosa (Fig. 2A). The observed inflammatory changes of the large intestine were associated with an increase in the weight of the colon (Fig. 3B). Treatment of rats with tempol resulted in a significant decrease in the extent and severity of the injury of the large intestine (Figs. 2B and 3B).

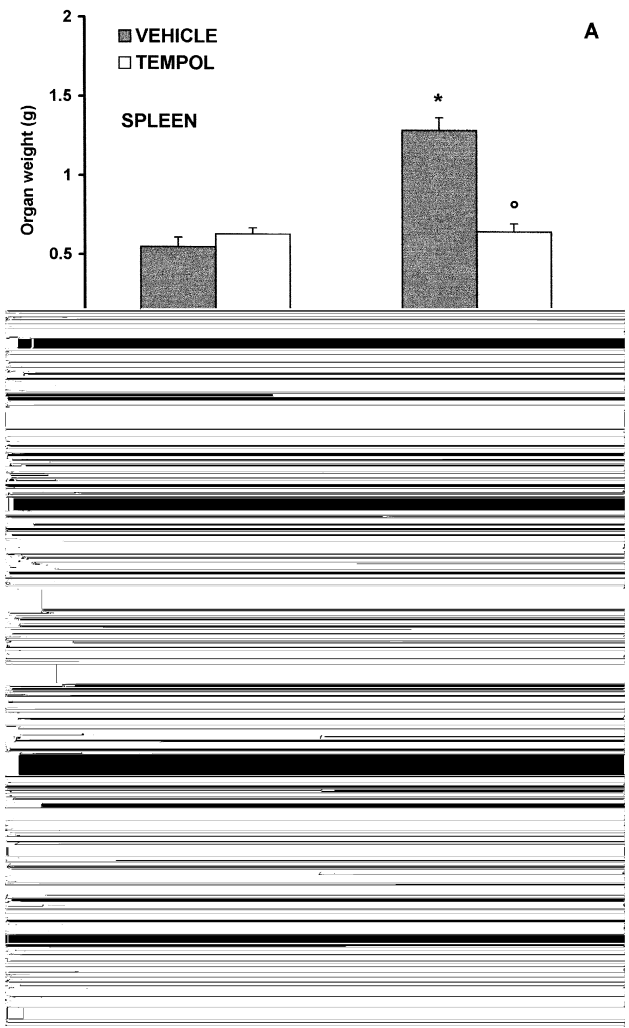

Fig. 3. Effect of tempol treatment on spleen (A) and colon (B) and body weight (C). A significant increase was consistently seen at 4 days after dinitrobenzene sulfonic acid injection in spleen (A) and colon (B). Tempol treatment significantly prevents the loss of body weight (C) as well as reduced the organ weight (A and B). Values are means ± S.E. of 15 rats for each group. $^{*}P < 0.01$ vs. sham; $^{\circ}P < 0.01$ vs. dinitrobenzene sulfonic acid.

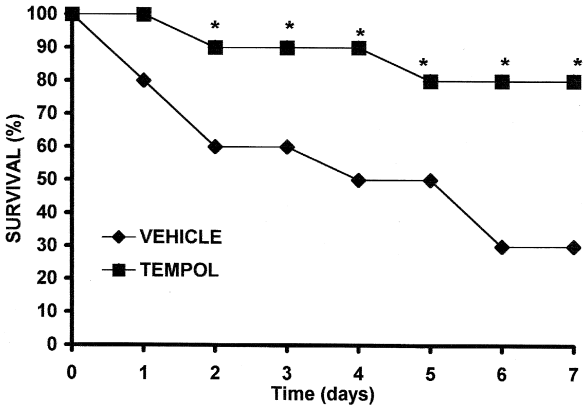

Fig. 4. Effect of tempol treatment on dinitrobenzene sulfonic acid-induced mortality. Survival is significantly improved in tempol-treated rats in comparison to the high mortality rate of the dinitrobenzene sulfonic acid-treated rat. $n = 10$ rats for each group. $^{*}P < 0.01$ vs. dinitrobenzene sulfonic acid.

The significant increased spleen weight also indicated the presence of inflammation (Fig. 3A). No significant increase of the weight was found in the colon and spleen of dinitrobenzene sulfonic acid-treated rats, which had been treated with tempol (Fig. 3A). Survival was monitored for 7 days. Dinitrobenzene sulfonic acid-treated rats developed bloody diarrhea and death occurred early and was substantial (45% and 70% died within 2 and 6 days, respectively, after dinitrobenzene sulfonic acid administration). In contrast, only 30% of tempol-treated rats had serious diarrhea and died (Fig. 4). The surviving rats appeared healthy and showed a very mild diarrhea.

The inflammatory changes of the intestinal tract were accompanied by a significant loss in body weight in comparison to control rats (Fig. 3C). Treatment with tempol significantly reduced the loss in body weight, which correlated well with the amelioration of colonic injury.

At 4 days after dinitrobenzene sulfonic acid treatment, colon sections were taken in order to determine the tissue localization of $ONOO^{-}$ and/or other nitrogen derivatives produced during colitis, nitrotyrosine, a specific marker of nitrosative stress (Beckman, 1996), was measured by immunohistochemical analysis in the distal colon. At 4 days after dinitrobenzene sulfonic acid treatment, colon sections were taken in order to determine the immunohistological staining for poly (ADP-ribose) synthetase. Sections of colon from sham-treated rats did not reveal any immunoreactivity for nitrotyrosine (Fig. 5A) and for poly (ADP-ribose) synthetase (Fig. 5B) within the normal architecture. A positive staining for nitrotyrosine (Fig. 5D) and for poly (ADP-ribose) synthetase (Fig. 5E) were found primarily localized in the infiltrated inflammatory cells and in disrupted epithelial cells.

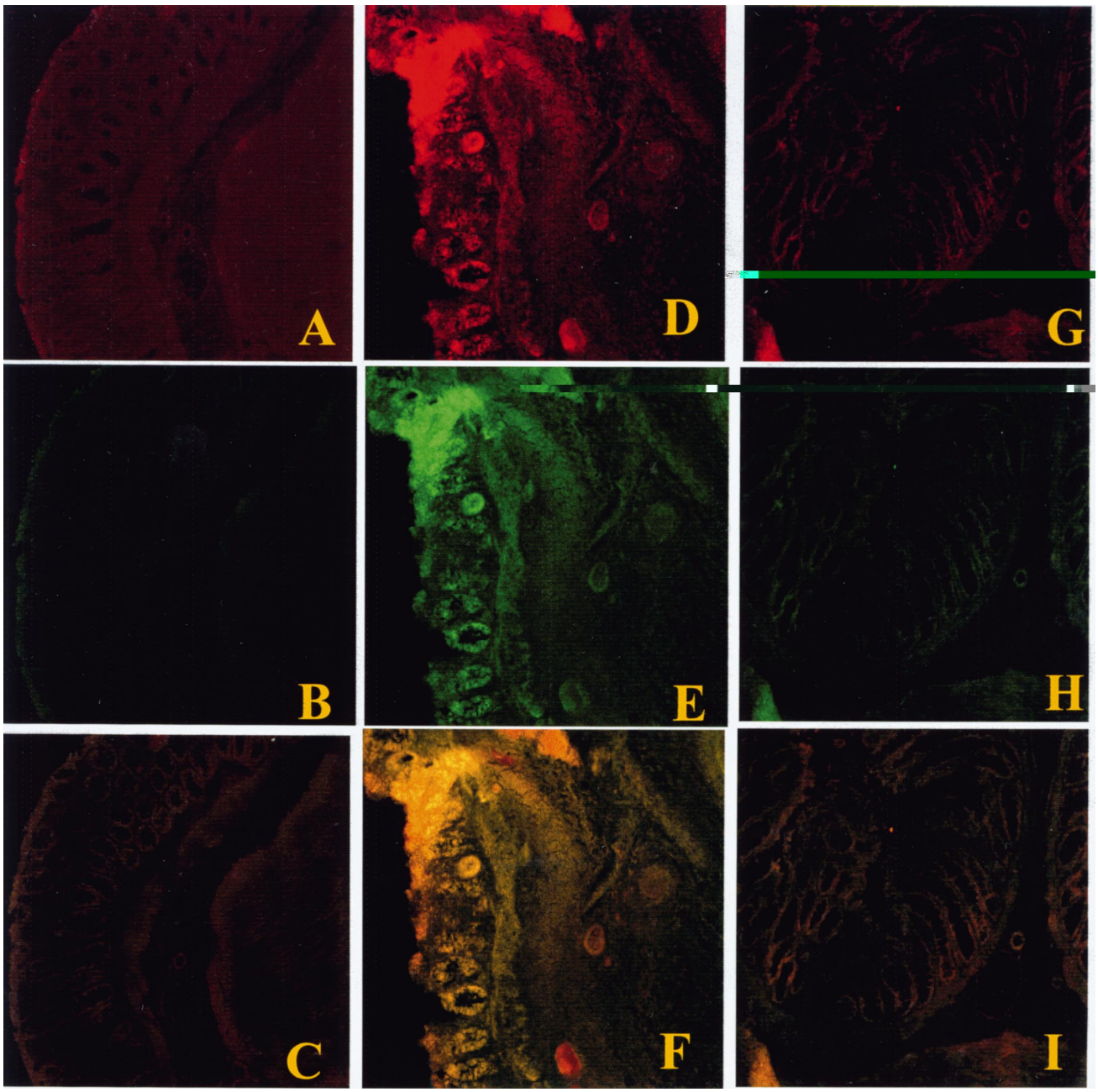

Fig. 5. Immunohistochemical localisation for nitrotytosine and for poly (ADP-ribose) synthetase in the colon. No positive staining for nitrotyrosine (A) and for poly (ADP-ribose) synthetase (B) was found in the colon section from sham-administered rats. Immunohistochemistry for nitrotyrosine (D) and for poly (ADP-ribose) synthetase (E) show positive staining localized in the injured area from dinitrobenzene sulfonic acid-treated rats. The intensity of the positive staining for nitrotyrosine (G) and for poly (ADP-ribose) synthetase (H) was significantly reduced in the ileum from tempol-treated rats. (C, F and I) represent the staining combination of panel A–B, D–E and G–H, respectively. Original magnification: ×100. Figure is representative of at least three experiments performed on different experimental days.

As demonstrated in Fig. 5F, the positive staining for nitrotyrosine and for poly (ADP-ribose) synthetase are co-localized in inflammatory cells and in disrupted epithelial cells. Tempol treatment reduced the degree of immunostaining for nitrotyrosine (Fig. 5G) and for poly (ADP-ribose) synthetase (Fig. 5H) in the colon of dinitrobenzene sulfonic acid-treated rats.

Colonic injury by dinitrobenzene sulfonic acid administration was also characterized by an increase in myeloperoxidase activity, indicative of neutrophil infiltration in inflamed tissue (Fig. 6A), confirming the enhanced leukocyte infiltration seen at histological inspection.

Infiltration of leukocytes into the mucosa has been suggested to contribute significantly to tissue necrosis and

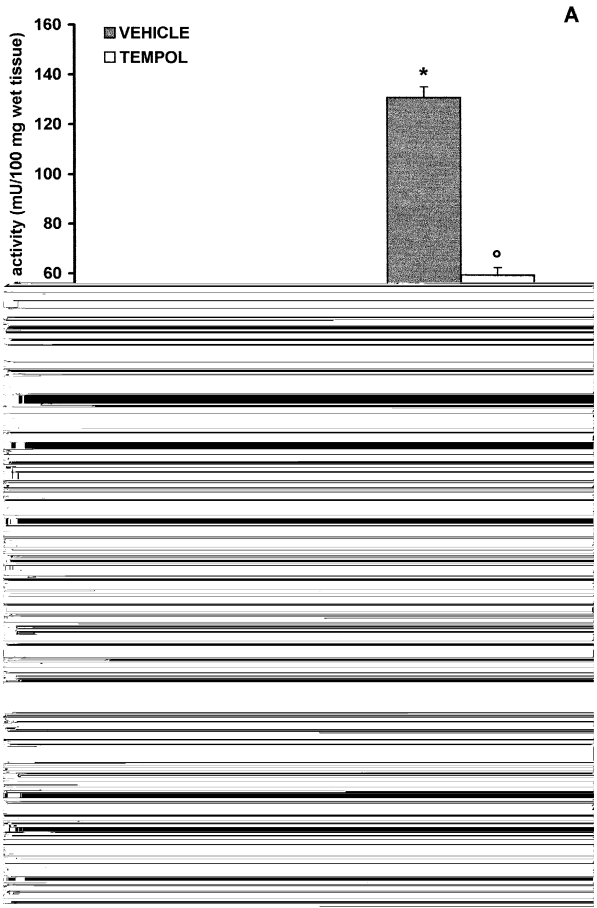

Fig. 6. Effect of tempol on neutrophil infiltration and lipid peroxidation. Myeloperoxidase activity (A) and malondialdehyde (B) in the colon from dinitrobenzene sulfonic acid-treated rats. Myeloperoxidase activity and malondialdehyde levels were significantly increased in dinitrobenzene sulfonic acid-treated rats in comparison to sham. Tempol-treated rats show a significant reduction of myeloperoxidase activity and malondialdehyde levels. Values are means ± S.E. of 15 rats for each group. * $P < 0.01$ vs. sham; ° $P < 0.01$ vs. dinitrobenzene sulfonic acid.

mucosal dysfunction of colitis by generating free radicals and oxidant molecules. In this study, the extent of myeloperoxidase activity closely paralleled the increase of tissue malondialdehyde, indicative of a massive lipid peroxidation (Fig. 6B). However, tempol treatment of dinitrobenzene sulfonic acid-treated rats significantly prevented neutrophil infiltration, as assessed by myeloperoxidase activity, and prevented the increased accumulation of malondialdehyde (Fig. 6).

To further elucidate the effect of tempol treatment on neutrophil accumulation in inflamed colon, we evaluated the intestinal expression of ICAM-1 and P-selectin. Tissue sections obtained from sham-operated rats with anti-ICAM-1 antibody showed a specific staining along the vessels, demonstrating that ICAM-1 is expressed constitutively in endothelial cells (Fig. 7A). After dinitrobenzene sulfonic acid 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 dinitrobenzene sulfonic acid-treated rats (Fig. 7D). Sections from tempol-treated rats did not reveal any upregulation of the constitutive ICAM-1, which was normally expressed in the endothelium along the vascular wall (Fig. 7G). No positive staining was observed in tissue sections obtained from sham-treated rats with anti-P-selectin antibody (Fig. 7B). Tissue section of the colon obtained from dinitrobenzene sulfonic acid-treated rats showed positive staining for P-selectin localized in the vascular endothelium (Fig. 7E). In tissue obtained from tempol-treated rats, no expression of P-selectin (Fig. 7H) was found. As can be seen in Fig. 7E, the positive staining for P-selectin and for ICAM-1 are co-localized in the in the endothelium.

4. Discussion

Inflammatory bowel diseases is a multi-factorial disorder of undetermined aetiology, which is characterised by a severe inflammation of the large intestine. We demonstrate in this study that dinitrobenzene sulfonic acid causes a substantial degree of inflammation and tissue injury (histology) in the rat colon, which is associated with an infiltration of the colon with polymorphonuclear cells (histology and myeloperoxidase activity) as well as lipid peroxidation. The degree of inflammation, tissue injury and lipid peroxidation caused by dinitrobenzene sulfonic was substantially reduced in rats treated with the membrane-permeable radical scavenger tempol. What then is the mechanism by which tempol protects the colon against the injury and inflammation caused by dinitrobenzene sulfonic acid? Tempol is a stable piperidine nitroxide (stable free radical) of low molecular weight and scavenges superoxide anions in vitro (Laight et al., 1997a,b). In addition, tempol inhibits the catalytic action of transition metal irons and hence, attenuates the formation of hydroxyl radicals (Lawrence and Spinger, 1991). Similarly, tempol protects cultured rabbit epithelial cells against the injury caused by hydrogen peroxide (Novelli, 1992; Laight et al., 1997a,b). Thus, tempol scavenges intracellular superoxide anions and prevents the formation of hydroxyl radicals. Unlike recombinant SOD, which is not able to cross biological membranes (Fridovich, 1995), tempol is a water-soluble, small molecule which permeates biological membranes and hence, will function as an intracellular scavenger of radicals. For instance, perfusion of isolated hearts of the rat with buffer containing tempol (2.5 mM for 75 min) results in an accumulation of tempol in the cytosolic fraction of these hearts (Monti et al., 1996). There is some controversy as to whether tempol and other stable nitroxides are genuine “SOD-mimetics” or act as stoichiometric scavengers of superoxide anions (Weiss et al., 1993; Krishna et al., 1996). Regardless of its precise

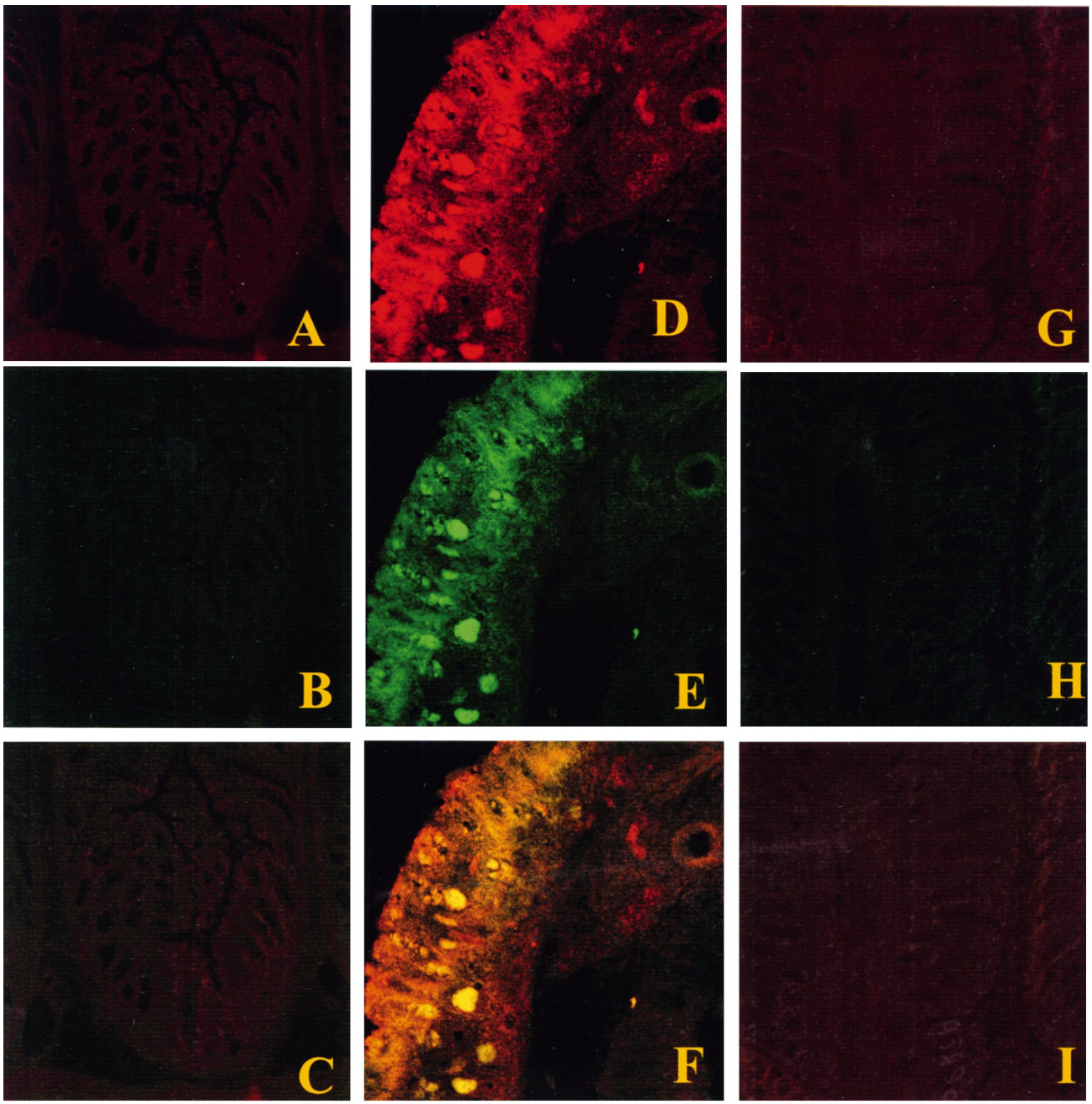

Fig. 7. Immunohistochemical localization of P-selectin in the colon. Staining of colon tissue sections obtained from sham-operated rats with anti-ICAM-1 antibody showed a specific staining along vessels, demonstrating that ICAM-1 is constitutively expressed (A). Ileum section from sham-operated rats revealed no positive for P-selectin staining (B). Section obtained from dinitrobenzene sulfonic acid-treated rats showed intense positive staining for ICAM-1 (D) and for P-selectin (E) on endothelial cells. The degree of endothelial staining for ICAM-1 (G) and for P-selectin (H) was markedly reduced in tissue section obtained from tempol-treated rats. (C, F and I) represent the staining combination of panel A–B, D–E and G–H, respectively. Original magnification: ×100. Figure is representative of at least three experiments performed on different experimental days.

mechanism(s) of action, there are many studies which document that tempol attenuates the effects of superoxide anions in vitro (Samuni et al., 1991; Reddan et al., 1993; Laight et al., 1997a,b). It has also been suggested that tempol reduces the formation of hydroxyl radicals generated in the Fenton reaction by reducing the intracellular levels of ferrous iron (Mitchell et al., 1990). Tempol also attenuates the impairment in mitochondrial respiration caused by hydrogen peroxide in rat cardiac myoblasts (McDonald et al., 1999). The decline in mitochondrial respiration caused by H_2O_2 in these cells (i) inversely correlates with the release of LDH and hence, cell death, and (ii) is prevented by iron chelators such as deferoxamine (Bowes et al., 1998, 1999). Thus, the cell injury and death caused by H_2O_2 in cardiac myoblasts of the rat is mediated by the generation of hydroxyl radicals (Bowes et

al., 1998, 1999). As tempol does not scavenge H_2O_2 (Reddan et al., 1993; Hahn et al., 1997), it is likely that tempol prevents the injury/death caused by hydrogen peroxide in rat cardiac myoblasts by preventing the effects of hydroxyl radicals. This hypothesis is supported by the following findings: Tempol (0.5 mM) also attenuates the impairment in contractility and reduces the release of high density lipoprotein caused by H_2O_2 in freshly isolated, cardiac myocytes of the rat and this injury is attenuated by deferoxamine (Samuni et al., 1991). The cell injury caused by hydrogen peroxide in rabbit lens epithelial cells is attenuated by deferoxamine (Reddan et al., 1993). We, therefore, suggest that the cardioprotective effects of tempol are due to the ability of this stable nitroxide radical to function as an intracellular scavenger of superoxide anions and hydroxyl radicals. One could, however, argue that the levels of tempol achieved in the rat in vivo are not sufficient to scavenge reactive oxygen. This is, however, not the case, as tempol attenuates the oxidation of dihydrorhodamine to rhodamine in rats subjected to haemorrhagic shock (McDonald et al., 1999). This method has been used in the past to document the generation of peroxynitrite and other reactive oxygen in the rat in vivo. In addition, tempol attenuates the degree of lipid peroxidation in the heart of rats subjected to haemorrhagic shock (McDonald et al., 1999). These data support the view that tempol acts as a radical scavenger in vitro and in vivo.

We also report in this study that tempol attenuates the increase in the number of polymorphonuclear cells in the colon of rats, which had received dinitrobenzene sulfonic acid to cause colitis. P-selectin is rapidly mobilised to the cell surface from preformed storage pools (located within endothelial cells) after exposure of the endothelium to, e.g. hydrogen peroxide, trombin, histamine or complement. Once in contact with the circulating blood, P-selectin facilitates the rolling of PMNs on the endothelial surface (first step in the adhesion/transmigration of polymorphonuclear cells) (Lawrence and Spinger, 1991; Geng et al., 1990). ICAM-1, which is constitutively expressed on the surface of endothelial cells, plays a pivotal role in the firm adhesion of polymorphonuclear cells to the endothelium (Butcher, 1993). Significant expression of ICAM-1 has been demonstrated in experimental colitis (Zingarelli et al., 1998). The expression/up-regulation of P-selectin and ICAM-1 is associated with the recruitment of polymorphonuclear cells to sites of inflammation (Clark et al., 1995). We found that the colitis caused by dinitrobenzene sulfonic acid in the rat was associated with a significant increase in the staining (of the colon by immunohistochemistry) for P-selectin and ICAM-1 on the endothelium of vessels located within the inflamed colon. Most notably, we report that tempol abolished the "up-regulation" of P-selectin and ICAM-1 (Figs. 5C and 6C), but did not affect the constitutive expression of ICAM-1 on endothelial cells (data not shown). The precise mechanism(s) by which Tempol prevents the upregulation of these proteins is not clear. It is possible that the prevention by Tempol of the formation of reactive oxygen attenuates the upregulation of P-selectin. It is also possible that any reduction by Tempol of the tissue injury caused by dinitrobenzene sulfonic acid will result in a weaker inflammatory response. Clearly, the absence of an increased expression of these adhesion molecules in the colon of tempol-treated rats correlated with the observed reduction in PMN infiltration as assessed by the determination of the tissue levels of the specific granulocyte enzyme myeloperoxidase. It is noteworthy, however, that tissue myeloperoxidase activity was not completely abolished. This result is consistent with previous studies demonstrating that constitutive levels of ICAM-1 appear to be sufficient to support a lower degree of CD11/CD18-dependent trans-endothelial migration of activated neutrophils (Furie et al., 1991).

We propose that the beneficial effect of tempol on organ integrity are due to the ability of this stable free nitroxide radical to function as an intracellular scavenger of superoxide anions and other free radical species. Indeed, this study clearly demonstrates that tempol largely attenuates the increase in lipid peroxidation associated with the colitis caused by dinitrobenzene sulfonic acid. The observed reduction of lipid peroxidation in the colon of tempol-treated rats was also associated with a reduction in the staining of the tissue for proteins containing nitrotyrosine moeities see below).

It is now well accepted that reactive oxygen including hydrogen peroxide, superoxide anions and hydroxyl radicals contributes to the tissue damage in inflammation. Inhibitors of NOS activity also reduce the tissue injury associated with inflammatory bowel diseases suggesting that — in addition to reactive oxygen — NO also plays an important role in the pathophysiology associated with this model of inflammation (Aiko and Grisham, 1995; Mourelle et al., 1996; Zingarelli et al., 1999b).

In addition to NO, $ONOO^-$ is also generated in inflammatory bowel diseases (Zingarelli et al., 1998, 1999a). We demonstrate here that injection of dinitrobenzene sulfonic acid leads to a substantial increase in the degree of nitrosylation of proteins in the colon. In contrast, the degree of staining for nitrotyrosine was significantly reduced in tempol-treated rats. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation (footprint) of $ONOO^-$ (Beckman, 1996). There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine (Eiserich et al., 1998). Increased nitrotyrosine staining is considered, therefore, as an indication of "increased nitrosative stress" rather than a specific marker of the generation of $ONOO^-$.

Reactive oxygen and $ONOO^-$ produce cellular injury and necrosis via several mechanisms including peroxida-

tion of membrane lipids, protein denaturation and DNA damage. Reactive oxygen produce strand breaks in DNA which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme poly (ADP-ribose) synthetase resulting in the depletion of its substrate NAD in vitro and a reduction in the rate of glycolysis. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed "the poly (ADP-ribose) synthetase Suicide Hypothesis" (Szabó et al., 1997). There is recent evidence that the activation of PARS may also play an important role in inflammation (Szabó et al., 1997, 1998; Cuzzocrea et al., 1998b,c; Zingarelli et al., 1999a). We also demonstrate here that the increase in poly (ADP-ribose) synthetase activity caused by dinitrobenzene sulfonic acid in the colon is attenuated in the colon of tempol-treated rats.

In conclusion, this study demonstrates that the degree of colitis caused by injection of dinitrobenzene sulfonic acid is significantly attenuated by the membrane-permeable radical scavenger tempol. The anti-inflammatory effects of tempol are associated with a reduction (i) the up-regulation of P-selectin and ICAM-1 leading to attenuation of the recruitment of neutrophils, (ii) lipid peroxidation, (iii) peroxynitrite formation, (iv) poly (ADP-ribose) synthetase activation and (v) ultimately tissue injury. Our findings suggest that tempol may be useful in colitis and other conditions associated with local or systemic inflammation.

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