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Inhibitors of poly (ADP-ribose) polymerase modulate signal transduction pathways in colitis

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Abstract

During inflammatory bowel diseases, oxidative and nitrosative stress induces DNA damage and activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP), resulting in depletion of intracellular energetics, intestinal barrier dysfunction and cellular death. The aim of our study was to evaluate the therapeutic efficacy of in vivo inhibition of PARP in experimental colitis, which was induced by rectal instillation of trinitrobenzene sulfonic acid (TNBS) in rats. In vehicle-treated rats, TNBS treatment resulted in colonic erosion and ulceration. Neutrophil infiltration (indicated by myeloperoxidase activity in the colon) was associated with formation of nitrotyrosine and marked apoptosis. Elevated levels of plasma nitrate/nitrite, metabolites of nitric oxide (NO), were also found. These inflammatory events were associated with the activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) in the colon; NF-κB was maximally activated at 3 and 7 days, whereas AP-1 increased 1 day after TNBS administration and declined thereafter. Treatment of the rats with the PARP inhibitors, 3-aminobenzamide or 1,5-dihydroxyisoquinoline, resolved colonic damage and reduced plasma levels of NO metabolites. Resolution of the damage was associated with reduction of neutrophil infiltration, nitrotyrosine formation and apoptosis. Treatment with PARP inhibitors also reduced DNA binding of NF-κB and AP-1 in the colon. These data demonstrate that pharmacological inhibition of PARP ameliorates colitis. Reduction of the inflammatory process is associated with modification of the activation of signal transduction pathways.

Keywords: Poly (ADP-ribose) polymerase; 3-Aminobenzamide; 1,5-Dihydroxyisoquinoline; NF-κB (nuclear factor-κB); Activator protein-1; Colitis

1. Introduction

Reactive oxygen species, such as superoxide anion and hydrogen peroxide, as well as nitrogen species, such as excess nitric oxide (NO) and peroxynitrite, have been implicated as mediators of inflammatory bowel diseases (Simmonds et al., 1992; Boughton-Smith et al., 1993; Grisham, 1994; Zingarelli et al., 1998, 1999a). Current evidence suggests that oxidative stress may function as an important signal for the upregulation of the genes of several pro-inflammatory mediators, which are known to promote alteration of the intestinal barrier function and cellular death by necrosis and/or apoptosis (Conner et al., 1996). Such a signal is mediated at the transcriptional level by a rapid activation of the enhancer elements nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Jourd'heuil et al., 1997; Karin et al., 1997; Baeuerle, 1998).

As highly reactive molecules, radicals and oxidants may indiscriminately attack DNA, causing oxidative modification and strand breakage (Cochrane, 1991). There is evidence that DNA from colonic biopsies of patients with ulcerative colitis has significantly increased levels of 8-hydroxyguanine, 2-hydroxyadenine, 8-hydroxyadenine and 2,6-diamino-5-formamido-pyrimidine (Wiseman and Halliwell, 1996; Lih-Brody et al., 1996). In cultured cells and in experimental animals, DNA damage has been shown to amplify cell dysfunction by activating the nuclear enzyme poly (ADP-ribose) polymerase (PARP). Excessive activation of PARP depletes the cellular pools of NAD^+, ATP and other high-energy phosphates leading to the loss of cell membrane integrity and viability. On the other hand, inhibition of PARP activity spares the cell from energy loss, preventing irreversible metabolic failure and thus providing cytoprotection (Cochrane, 1991; Zhang et al., 1994; Zingarelli et al., 1996). Furthermore, numerous studies have

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confirmed that activation of PARP is a major cytotoxic pathway of tissue injury in different pathologies associated with inflammation (Szabó and Dawson, 1998). In this regard, we have previously demonstrated that mice with a targeted deletion of the PARP gene are significantly more resistant to hapten-induced colitis than their control wild-type littermates, therefore suggesting that PARP plays a detrimental role in the development and persistence of tissue damage in colitis (Zingarelli et al., 1999b). It has been proposed that PARP activation also plays a critical role in the regulation of transcription, possibly by participating in the repair of DNA strand breaks or by catalyzing the poly (ADP-ribosyl)ation of transcription factors or post-translation proteins (De Murcia et al., 1988; Vispé et al., 2000).

Consistent with these findings, the objective of the present study was to investigate the biological effects of pharmacological inhibition of PARP in a rat model of trinitrobenzene sulfonic acid (TNBS)-induced colitis. To this aim, two structurally unrelated selective inhibitors of PARP, 3-aminobenzamide and 1,5-dihydroxyisoquinoline, which show high specificity and selectivity for PARP (Banasik et al., 1992; Zingarelli et al., 1997; Chatterjee et al., 2000), were tested. We observed that pharmacological inhibition of PARP activity resulted in a faster resolution of colonic inflammation, and that this therapeutic efficacy was associated with prevention of NF-κB and AP-1 activation.

2. Materials and methods

2.1. Animals

Male Wistar rats (Charles River Laboratories, Wilmington, MA), weighing 250–300 g, were housed in a room with controlled temperature (22 °C) and 12-h light/dark cycle. The animals were food fasted 24 h before experimentation and allowed food and water ad libitum after the induction of colitis.

2.2. Induction of colitis

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by US National Institutes of Health (NIH Publication No. 85-23 revised 1996) and with the approval of the Institutional Animal Care and Use Committee. Colitis was induced by using a technique of hapten-induced colonic inflammation as previously described (Zingarelli et al., 1998). In fasted rats lightly anaesthetized with isoflurane, a 3.5 F catheter was inserted into the colon via the anus until approximately the splenic flexure (8 cm from the anus). 2,4,6-Trinitrobenzene sulfonic acid (TNBS, 20 mg/rat) was dissolved in 50% ethanol (vol/vol) and injected (0.25 ml) into the colon via the rubber cannula. Animals were then kept in a vertical position for 30 s and returned to their cages. Four groups of rats were used in the experiment. The first group ($n=12$) received an equal volume of vehicle (0.9% NaCl solution) instead of the PARP inhibitors (TNBS + vehicle group). The second and third groups ($n=12$ for each group) received 3-aminobenzamide (10 mg/kg) or 1,5-dihydroxyisoquinoline (1 mg/kg) by intraperitoneal injection twice a day. A fourth group of animals ($n=4$, group at time 0) was sacrificed without receiving TNBS or PARP inhibitors. In comparative experiments, the effect of PARP inhibitors was tested in groups of animals which received intracolonic administration of 50% ethanol alone. The dosage of PARP inhibitors regimen has been previously shown to exert anti-inflammatory effects (Zingarelli et al., 1997; Chatterjee et al., 2000). Drug treatment was started 24 h before TNBS intracolonic administration and continued until the end of the experimental period (7 days after TNBS administration). Animals were sacrificed at 1, 3 and 7 days after TNBS administration and a segment of the colon 8 cm long was excised for the evaluation of macroscopic damage. Tissue segments 1 cm in length were then fixed in 10% buffered formalin or immediately frozen in liquid nitrogen and stored at −70 °C for the histological, immunohistochemical and biochemical studies described below.

2.3. Evaluation of colonic damage

Colonic damage was scored by two independent observers as previously described (Morris et al., 1989; Zingarelli et al., 1998), according to the following morphological criteria: score 0, no damage; score 1, localized hyperemia without ulcers; score 2, linear ulcers with no significant inflammation; score 3, linear ulcers with inflammation at one site; score 4, two or more major sites of ulceration and/or inflammation; score 5, two or more sites of inflammation and ulceration extending >1 cm along the length of the colon; score 6–10, one point is added for each cm of ulceration beyond an initial 2 cm.

2.4. Histopathological analysis

For microscopic histological evaluation, formalin-fixed tissues were embedded in paraffin and 5-μm sections were stained with hematoxylin and eosin and evaluated by light microscopy by a pathologist blinded to the experimental protocol.

2.5. Determination of apoptosis

Cell death by apoptosis in the inflamed colon was evaluated by measurement of oligonucleosomal DNA fragments by a histochemical TdT-mediated dUTP nick-end labeling (TUNEL)-like staining (TdT-FragEL kit, Oncogene Research Products, Cambridge, MA). Briefly, paraffin-embedded sections, after deparaffination, were permeabilized with protease K (2 mg/ml) in 10 mM Tris (pH 8) at room temperature for 20 min. Endogenous peroxidase was

quenched with 3% H_2O_2 in methanol for 5 min. Sections were incubated with a reaction buffer composed by biotin-dCTP and unlabeled dCTP and TdT enzyme (terminal deoxynucleotidyl transferase) in a humidified chamber at 37 °C. In this assay, TdT binds to exposed 3′OH ends of DNA fragments and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinilated nucleotides were then detected using a streptavidin–horseradish peroxidase conjugate and diaminobenzidine (Gavrieli et al., 1992).

2.6. Measurement of nitrite/nitrate production

Nitrite/nitrate production, an indicator of NO synthesis, was measured in plasma samples as previously described (Zingarelli et al., 1999a). Nitrate in the plasma was reduced to nitrite by incubation for 3 h with nitrate reductase (670 mU/ml) and NADPH (160 mM) at room temperature. Nitrite concentration in the samples was then measured by the Griess reaction by adding 100 μl of Griess reagent (0.1% naphthalethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 5% concentrated H_3PO_4; vol 1:1) to 100-μl samples. The optical density at 550 nm (OD_{550}) was measured using a Spectramax 250 microplate reader (Molecular Devices Sunnyvale, CA). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrate prepared in saline solution.

2.7. Assay of myeloperoxidase activity

Myeloperoxidase activity was determined as an index of neutrophil accumulation (Krawisz et al., 1984). Colonic tissues were homogenized 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 by spectrophotometry at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 37 °C and was expressed in milliunits per 100-mg weight of tissue.

2.8. Immunohistochemistry for nitrotyrosine

Tyrosine nitration was detected in colonic sections by immunohistochemistry (Zingarelli et al., 1998). Frozen sections 5 μm thick were fixed in 4% paraformaldehyde and incubated for 2 h with a blocking solution (0.1 M phosphate buffered saline containing 0.1% Triton X-100 and 2% normal goat serum) in order to minimize non-specific adsorption. Sections were then incubated overnight with 1:750 dilution of primary anti-nitrotyrosine antibody or with control solutions. Controls included buffer alone or non-specific purified rabbit immunoglobulin G (IgG). Specific labeling was detected by incubating for 30 min with a biotin-conjugated goat anti-rabbit IgG and amplified with avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) after quenching endogenous peroxidase with 0.3% H_2O_2 in 100% methanol for 15 min. Diaminobenzidine was used as a chromogen.

2.9. Nuclear extraction

Tissue samples from colons were homogenized with a Polytron homogenizer in a buffer containing 0.32 M sucrose, 10 mM Tris–HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 5 mM NaN_3, 10 mM β-mercaptoethanol, 20 μM leupeptin, 0.15 μM pepstatin A, 0.2 mM phenylmethanesulfonyl fluoride, 50 mM NaF, 1 mM sodium orthovanadate, 0.4 nM microcystin. The homogenates were centrifuged (1000 × *g*, 10 min) and the pellets were solubilized in Triton buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 20 μM leupeptin A, 0.2 mM phenylmethanesulfonyl fluoride). The lysates were centrifuged (15,000 × *g*,

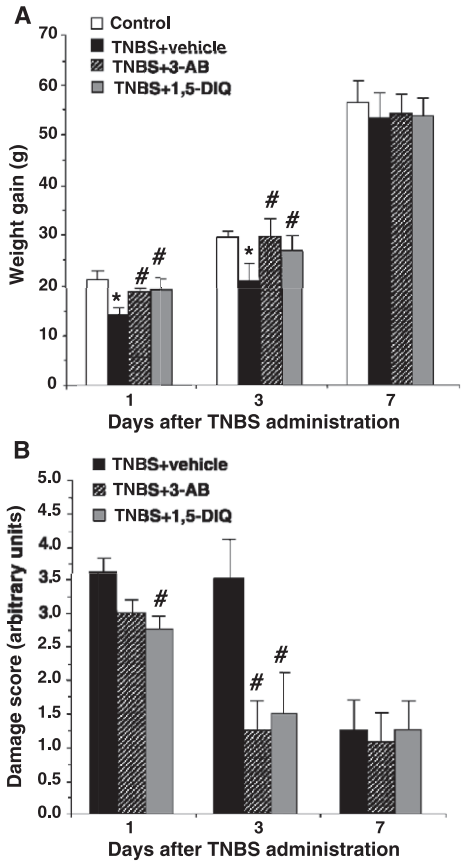

Fig. 1. Effect of in vivo treatment with 3-aminobenzamide (3-AB) or 1,5-dihydroxyisoquinoline (1,5-DIQ) on weight gain (A) and colon damage (B) in rats subjected to TNBS-induced colitis. Each data point represents the mean ± S.E.M. of 4–6 animals for each group. * Represents $P<0.05$ vs. sham rats (control); # represents $P<0.05$ vs. vehicle-treated rats subjected to colitis (TNBS + vehicle).

30 min, 4 °C), and the supernatant (nuclear extract) was collected.

2.10. Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were performed as previously described (Zingarelli et al., 2001). Oligonucleotide probes corresponding to NF-κB consensus sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) or to AP-1 consensus sequence (5′-CGC TTG ATG ACT CAG CCG GAA-3′) were labeled with γ-[^{32}P]ATP using T4 polynucleotide kinase and purified in Bio-Spin chromatography columns (BioRad, Hercules, CA). Nuclear protein (10 μg) was pre-incubated with EMSA buffer (12 mM HEPES pH 7.9, 4 mM Tris–HCl pH 7.9, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 50 ng/ml poly [d(I-C)], 12% glycerol v/v and 0.2 mM phenylmethanesulfonyl fluoride) on ice for 10 min before addition of the radiolabeled oligonucleotide for an additional 10 min. Protein–nucleic acid complexes were resolved using a non-denaturing polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide/bisacrylamide) and run in 0.5× TBE (45 mM Tris–HCl, 45 mM boric acid, 1 mM EDTA) for 1 h at constant current (30 mA). Gels were transferred to Whatman 3 M paper, dried under a vacuum at 80 °C for 1 h and exposed to photographic film at −70 °C with an intensifying screen. Densitometric analysis was performed using ImageQuant (Molecular Dynamics, Sunnyvale, CA).

2.11. Materials

Primary anti-nitrotyrosine antibody was purchased from Upstate Biotech (Saranac Lake, NY). The oligonucleotide probes for NF-κB and AP-1 consensus were obtained from

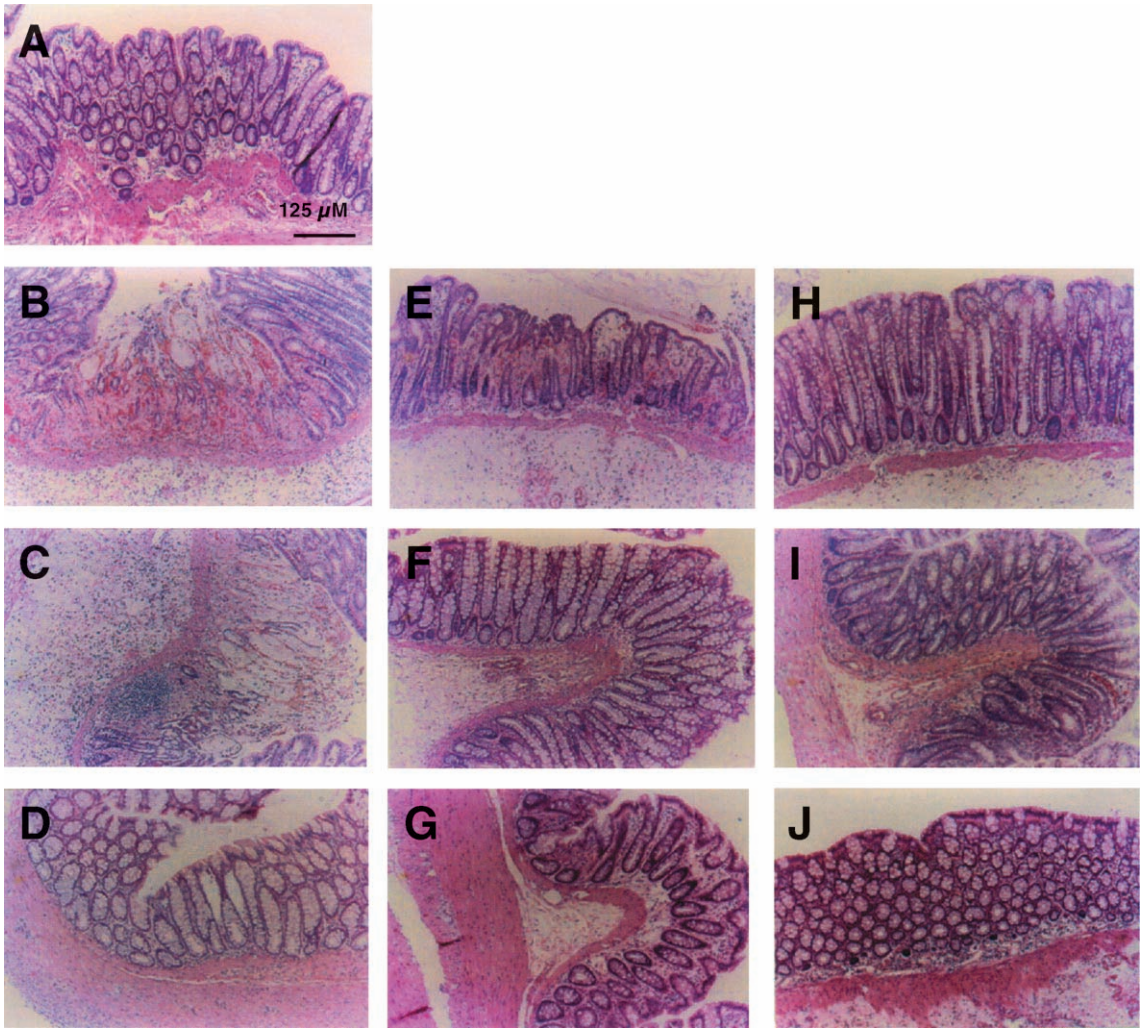

Fig. 2. Effect of in vivo treatment with 3-aminobenzamide or 1,5-dihydroxyisoquinoline on time course of changes in colonic architecture after TNBS administration. (A) Representative colonic sections from a control rat showed normal architecture at day 0. At day 1 (B) and 3 (C) after TNBS administration, a marked disruption of the epithelium was associated with a massive infiltration of inflammatory cells. At day 7 (D), a healing process started in the epithelium. Colonic damage was reduced in rats treated with 3-aminobenzamide (E, day 1; F, day 3; G, day 7) or 1,5-dihydroxyisoquinoline (H, day 1; I, day 3; J, day 7). Magnification: × 100. A similar pattern was seen in 3–4 different tissue sections in each experimental group.

Santa Cruz Biotechnology (Santa Cruz, CA). Reagents, secondary and non-specific IgG antibodies for immunohistochemical analysis were from Vector Laboratories (Burlingame, CA). The compound 1,5-dihydroxyisoquinoline was purchased from Alexis Biochemicals (San Diego, CA). All other chemicals were from Sigma/Aldrich (St. Louis, MO).

2.12. Data analysis

All values in the figures and text are expressed as mean ± standard error of the mean of n observations, where n represents the number of rats ($n = 3–6$ animals for each group). Data sets were examined by one- and two-way analysis of variance, and individual group means were compared with Student's unpaired t-test. Statistical analysis of scores was performed using the Mann–Whitney U-test. A P-value less than 0.05 was considered significant.

3. Results

3.1. Effect of PARP inhibition on the severity of colitis

Animals were subjected to intraluminal administration of TNBS (20 mg/rat) in 50% ethanol, a mixture that consistently induces colonic epithelial injury with ulceration (Morris et al., 1989). In comparative experiments, rats were treated with 50% ethanol only, which did not cause any macroscopic damage. Signs of illness were monitored for 7 days. As early as 24 h, vehicle-treated rats exhibited a serious bloody diarrhea, which was associated with a reduction of weight gain (Fig. 1A). In contrast, rats, which were treated with 3-aminobenzamide or 1,5-dihydroxyisoquinoline, appeared healthy, exhibited a very mild diarrhea and gained weight similar to rats receiving intracolonic administration of ethanol only (Fig. 1A).

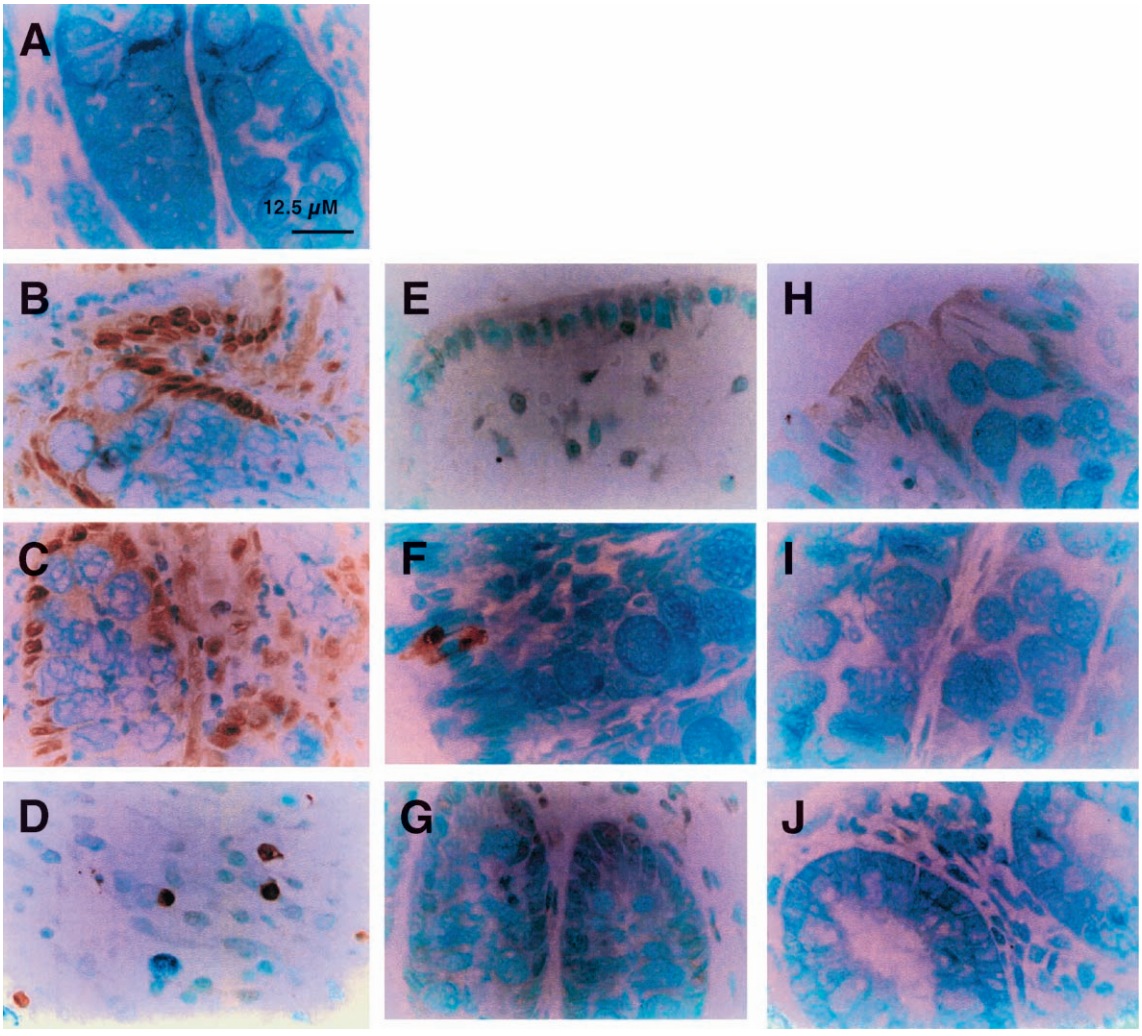

Fig. 3. Effect of in vivo treatment with 3-aminobenzamide or 1,5-dihydroxyisoquinoline on time course of changes in colonic apoptosis after TNBS administration. (A) Representative colonic sections from a sham rat showed negative staining (day 0). At day 1 (B) and 3 (C) after TNBS administration, a brown staining revealed the presence of apoptotic nuclei and intercellular apoptotic fragments in the epithelium and infiltrated inflammatory cells. At day 7 (D), apoptotic inflammatory cells were still present in the submucosa. Apoptosis staining was reduced in rats treated with 3-aminobenzamide (E, day 1; F, day 3; G, day 7) or 1,5-dihydroxyisoquinoline (H, day 1; I, day 3; J, day 7). Magnification: × 1000. A similar pattern was seen in 3–4 different tissue sections in each experimental group.

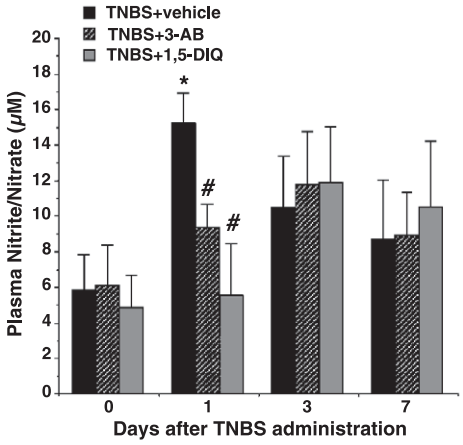

Fig. 4. Effect of in vivo treatment with 3-aminobenzamide (3-AB) or 1,5-dihydroxyisoquinoline (1,5-DIQ) on plasma levels of nitrite/nitrate in rats subjected to TNBS-induced colitis. Each data point represents the mean ± S.E.M. of 4–6 animals for each group. * Represents $P < 0.05$ vs. control rats at day 0; # represents $P < 0.05$ vs. vehicle-treated rats subjected to colitis (TNBS + vehicle).

Macroscopic inspection of the distal colon and rectum at 24 h after TNBS treatment demonstrated the presence of multiple sites of mucosal congestion, erosion and hemorrhagic ulcerations in vehicle-treated rats. The damage was still maintained at 3 days and appeared to be resolved at 7 days after TNBS administration. However, in vivo treatment with the PARP inhibitors reduced tissue damage, which resolved completely at 3 days (see Fig. 1B for damage score).

Histological examination of the colon confirmed the macroscopic findings. Erosions, edema, hemorrhage and large stretches of denuded epithelia were present at 1 and 3 days after TNBS administration in the colon of vehicle-treated rats. Alteration of colonic architecture was associated with a diffuse leukocyte infiltrate in the submucosa. At 7 days, a re-epithelization process was observed in the mucosa associated with some mild infiltrate in the submucosa (Fig. 2). In contrast, in vivo treatment with the PARP inhibitors markedly reduced erosions of the mucosa at 1 day after TNBS administration. At 3 and 7 days, the histological features of the colon were typical of normal or healing mucosa with an intact epithelium and no cellular infiltration (Fig. 2).

3.2. Effect of PARP inhibition on apoptosis in inflamed colon

To test whether tissue damage was associated with cell death by apoptosis, we measured oligonucleosomal DNA fragmentation in the inflamed colon. Almost no apoptotic cells were detectable in the colon of control rats (i.e. not subjected to TNBS administration). At 1 and 3 days after TNBS administration, tissues obtained from vehicle-treated rats demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments mostly localized in the deranged epithelium and in infiltrated neutrophils. At 7 days, apoptosis persisted mainly in some infiltrated neutrophils in the submucosa. In contrast, tissues obtained from rats treated with the PARP inhibitors demonstrated a small number of apoptotic cells or fragments organized at the surface of the epithelial mucosa (Fig. 3).

3.3. Effect of PARP inhibition on nitrite/nitrate production

Production of NO from the inducible NO synthase (iNOS) during colitis has been suggested to contribute

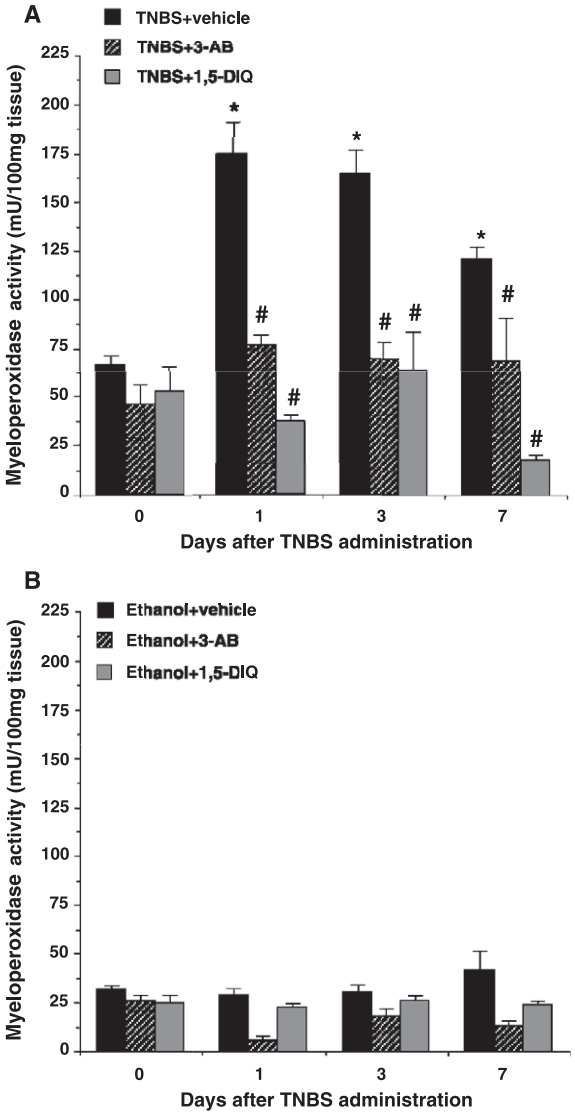

Fig. 5. Effect of in vivo treatment with 3-aminobenzamide (3-AB) or 1,5-dihydroxyisoquinoline (1,5-DIQ) on myeloperoxidase activity in rats subjected to TNBS-induced colitis (A) and in rats subjected to administration of 50% ethanol only (B). Myeloperoxidase, an enzyme present in neutrophils, was measured as an index of neutrophil infiltration into the injured tissue. Each data point represents the mean ± S.E.M. of 4–6 animals for each group. * Represents $P < 0.05$ vs. control rats at day 0; # represents $P < 0.05$ vs. vehicle-treated rats subjected to colitis (TNBS + vehicle).

significantly to tissue damage (Boughton-Smith et al., 1993; Grisham, 1994; Zingarelli et al., 1998, 1999a). Therefore, we next determined the effect of in vivo treatment with 3-aminobenzamide or 1,5-dihydroxyisoquinoline on NO production. In vehicle-treated rats, plasma levels of nitrite/nitrate, stable metabolites of NO, increased as early as 1 day after TNBS administration and declined thereafter. Treatment with the PARP inhibitors reduced this early increase (Fig. 4).

3.4. Effect of PARP inhibition on neutrophil infiltration and nitrotyrosine formation

Colonic injury in vehicle-treated rats was also characterized by an increase in myeloperoxidase activity, indicative of neutrophil infiltration into the inflamed tissue (Fig. 5A), which confirmed the very large influx of leukocytes observed in histologic specimens. Infiltration of leukocytes into the mucosa has been suggested to contribute significantly to the epithelial dysfunction of colitis by releasing free oxygen and nitrogen radicals and favoring the formation of peroxynitrite (Grisham, 1994; Zingarelli et al., 1998, 1999a). In our study, immunohistochemistry for nitrotyrosine revealed the occurrence of nitrosylated proteins in colons of vehicle-treated rats at 1 and 3 days after TNBS administration. The staining for nitrotyrosine was found on epithelial and infiltrated inflammatory cells, and along the vessels of the villi and lamina propria, and decreased by 7 days after TNBS administration (Fig. 6). However, pharmacological inhibition of PARP prevented neutrophil infiltra-

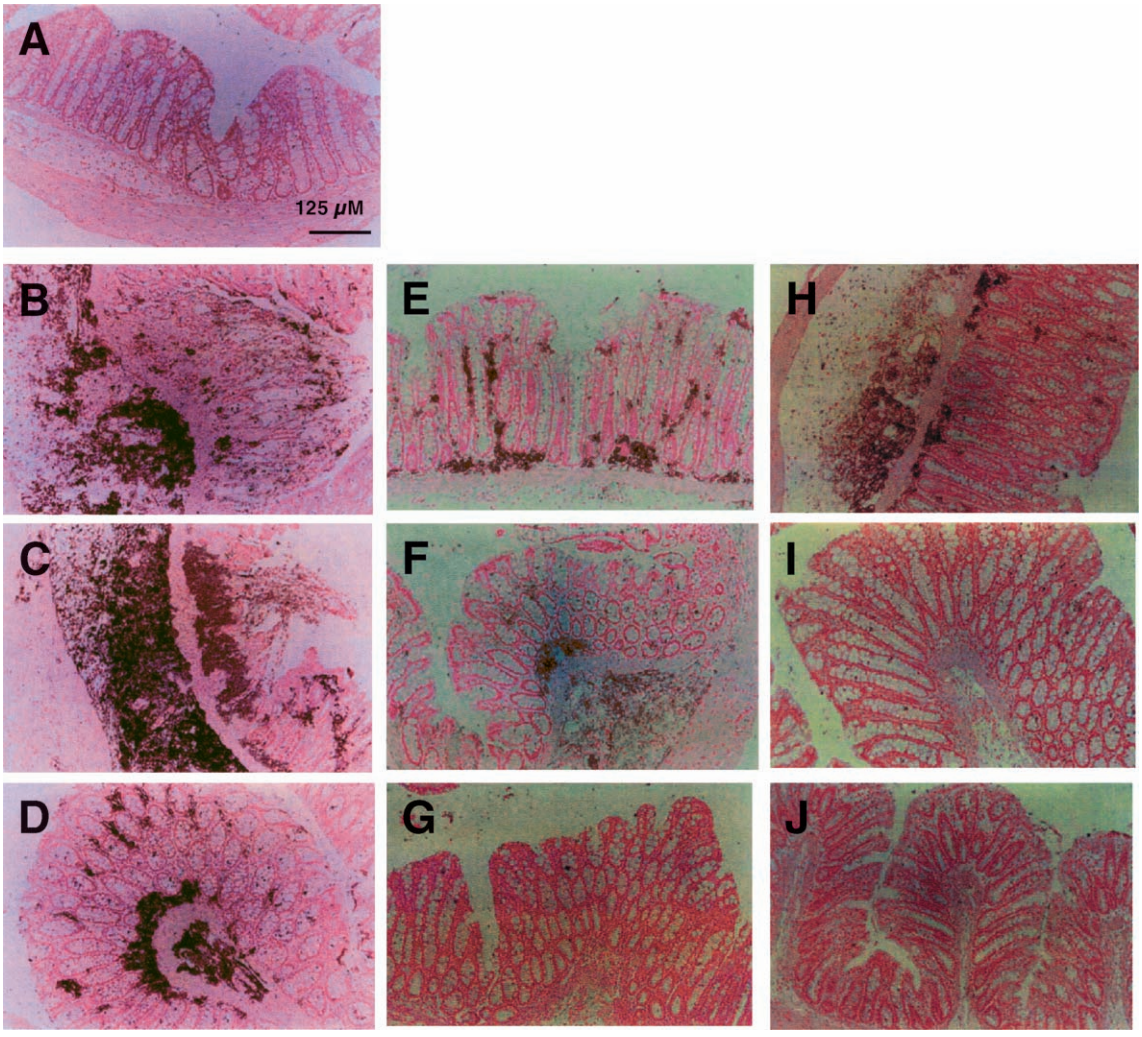

Fig. 6. Representative immunostaining of nitrotyrosine, as a marker of nitrosative stress, in the colon. (A) Representative colonic sections from a control rat showed negative staining or non-specific background for nitrotyrosine at day 0. At day 1 (B) and 3 (C) after TNBS administration, a diffuse dark staining was localized mainly in the deranged epithelium, submucosa and infiltrated inflammatory cells. At day 7 (D), reduced nitrotyrosine staining was still present in the area of healing mucosa, and in some vessels of the villi and lamina propria. Nitrotyrosine staining was reduced in rats treated with 3-aminobenzamide (E, day 1; F, day 3; G, day 7) or 1,5-dihydroxyisoquinoline (H, day 1; I, day 3; J, day 7). Magnification: × 100. A similar pattern was seen in 3–4 different tissue sections in each experimental group.

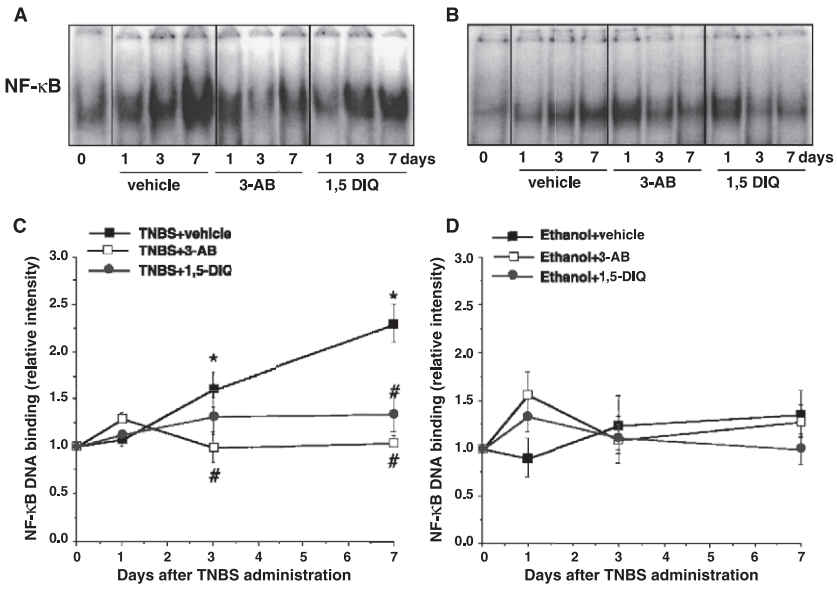

Fig. 7. Effect of in vivo treatment with 3-aminobenzamide (3-AB) or 1,5-dihydroxyisoquinoline (1,5-DIQ) on the activation of NF-κB in the colon of rats subjected to TNBS-induced colitis (A and C) and rats subjected to administration of 50% ethanol only (B and D). (A and B) Representative autoradiograph of electrophoretic mobility shift assay for NF-κB. (C and D) Image analysis of activation of NF-κB determined by densitometry from the autoradiograph. Results are representative of three separate time-course experiments. * Represents $P < 0.05$ vs. respective control value (time 0). Fold increase was calculated vs. respective control value (time 0) set to 1.0.

tion, as assessed by myeloperoxidase activity (Fig. 5A), and reduced the nitrosylation of proteins (Fig. 6). In comparative experiments, no significant increase of infiltrated neutrophils was observed in rats receiving intracolonic administration of ethanol only (Fig. 5B).

3.5. Effect of PARP inhibition on activation of NF-κB and AP-1

To investigate the cellular mechanisms by which treatment with 3-aminobenzamide or 1,5-dihydroxyisoquinoline

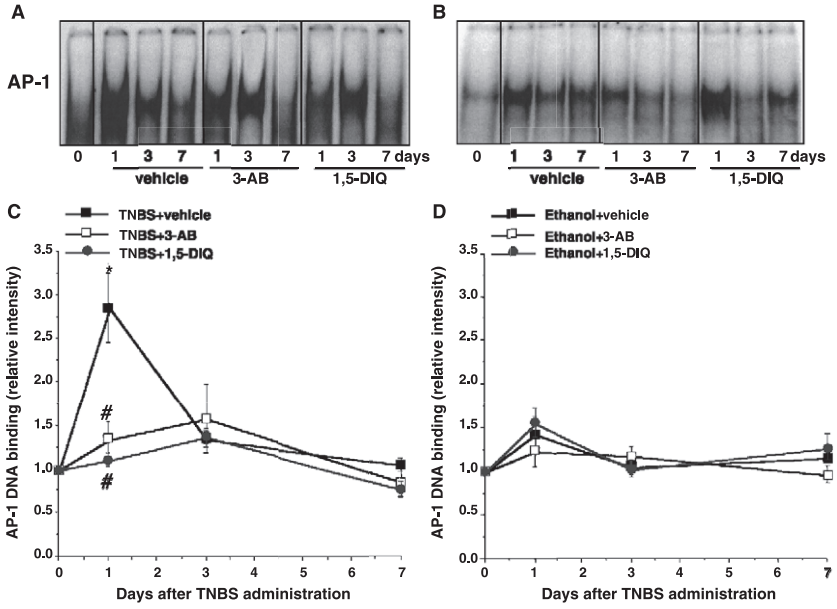

Fig. 8. Effect of in vivo treatment with 3-aminobenzamide (3-AB) or 1,5-dihydroxyisoquinoline (1,5-DIQ) on the activation of AP-1 in the colon of rats subjected to TNBS-induced colitis (A and C) and rats subjected to administration of 50% ethanol only (B and D). (A and B) Representative autoradiograph of electrophoretic mobility shift assay for AP-1. (C and D) Image analysis of activation of AP-1 determined by densitometry from the autoradiograph. Results are representative of three separate time-course experiments. * Represents $P < 0.05$ vs. respective control value (time 0). Fold increase was calculated vs. respective control value (time 0) set to 1.0.

may attenuate TNBS-induced injury, we evaluated the nuclear binding of NF-κB and AP-1, two major transcription factors involved in the signal transduction of inflammation (Karin et al., 1997; Baeuerle, 1998). In a time course study, we found that in vehicle-treated rats, nuclear DNA binding of NF-κB increased steadily in a time-dependent manner reaching a peak at 7 days after TNBS administration (Fig. 7). DNA binding of AP-1 increased as early as 1 day after TNBS administration and declined thereafter (Fig. 8). Treatment with the PARP inhibitors reduced DNA binding activity of both NF-κB and AP-1 (Figs. 7 and 8). In comparative experiments, in the colon of rats receiving ethanol only, there was a slight but not significant increase of the nuclear binding of NF-κB and AP-1 after intracolonic administration of ethanol when compared to time 0. The nuclear binding of NF-κB and AP-1 exhibited similar kinetics as those observed in the colon of rats receiving intracolonic administration of TNBS and was not significantly modified by treatment with the PARP inhibitors (Figs. 7 and 8).

4. Discussion

In this report we demonstrate that pharmacological inhibition of PARP exerts beneficial effects in a rat model of colitis induced by the intracolonic administration of TNBS. We observed that treatment with 3-aminobenzamide or 1,5-dihydroxyisoquinoline, two structurally unrelated selective inhibitors of PARP, significantly decreased colon damage, cell apoptosis, tissue sequestration of neutrophils and formation of nitrotyrosine. These protective effects were associated with inhibition of DNA binding of the transcription factors NF-κB and AP-1 in the inflamed colon. Thus, we propose that the anti-inflammatory activity of PARP inhibitors may be mediated, at least in part, by inhibition of the transcription of certain pro-inflammatory mediators, which are regulated by NF-κB and AP-1 to varying degrees. Together with our previous finding that genetic deletion of PARP renders mice more resistant to TNBS-induced colitis (Zingarelli et al., 1999b), this current study further supports the hypothesis that PARP plays a detrimental role in the development and persistence of colitis and may represent a therapeutic target.

Inflammatory bowel disease is a heterogeneous disorder with multiple etiologic and pathogenic mechanisms (Fiocchi, 1998). A central feature of its pathophysiology is an exaggerated release of oxygen and nitrogen-reactive species (Simmonds et al., 1992; Boughton-Smith et al., 1993; Grisham, 1994; Zingarelli et al., 1998, 1999a) and an overproduction of pro-inflammatory mediators (Fiocchi, 1998). Extensive experimental studies have shown that a variety of these pro-inflammatory genes (i.e. cytokines, iNOS and adhesion molecules) are regulated at the transcription level by the nuclear factors NF-κB and/or AP-1 (Jourd'heuil et al., 1997; Karin et al., 1997; Baeuerle, 1998). Of clinical relevance, it has been found that lamina propria macrophages from patients with Crohn's disease and ulcerative colitis display high levels of NF-κB binding activity accompanied by an increased production of interleukin-1, interleukin-6 and tumour necrosis factor-α (TNF-α) (Neurath et al., 1998). NF-κB DNA binding activity has also been found to increase in the inflamed mucosa of colon biopsies in patients with active inflammatory bowel disease and to correlate with severity of the disease (Schreiber et al., 1998; Ardite et al., 1998). A few reports have also demonstrated a role for AP-1 activation in intestinal inflammation. Gonsky et al. (1998) have demonstrated that activation of lamina propria T cells from normal, ulcerative colitis, or Crohn's disease mucosa through the CD2 pathway leads to induction of AP-1 complexes that bind to the interleukin-2 promoter. Transcriptional activation of AP-1 is enhanced in immunostimulated human colonic epithelial cells (Abreu-Martin et al., 1999). In addition, we have previously demonstrated that AP-1 DNA binding is increased during inflammation of small bowel induced by vascular impairment in interleukin-10-deficient mice (experimental animals prone to develop inflammatory bowel disease) (Zingarelli et al., 2001). In our experimental model of colitis, we found that DNA binding activity of both NF-κB and AP-1 is increased after TNBS administration. Interestingly, the changes in AP-1 DNA binding occurred at an earlier stage (i.e. 1 day after TNBS administration) and correlated with a more severe degree of inflammation. Instead, NF-κB DNA binding activity steadily increased over time up to 7 days after TNBS administration, paralleling both the acute period of inflammation and the initiation of the healing process. Whether prolonged activation of NF-κB may also maintain the inflammatory process of chronic colitis needs further investigation. Nevertheless, our data support the well-established hypothesis that NF-κB may represent an important therapeutic target in the treatment of human inflammatory bowel disease. In this regard, many therapeutic agents used to treat patients with Crohn's disease and ulcerative colitis, including sulfasalazine (Wahl et al., 1998) and steroids (Wissink et al., 1998; Ardite et al., 1998), have been shown to mediate their effects by acting as NF-κB inhibitors. Our present data show that the amelioration of colonic injury afforded by pharmacological inhibition of PARP was associated with inhibition of NF-κB and AP-1 DNA binding activity.

Although it is difficult to establish the definitive mechanism by which the PARP inhibitors abolish the DNA binding of these nuclear factors in in vivo experiments, our data support the possibility that PARP may be an important modulator of transcription during inflammation. Our findings are supported by other reports demonstrating a role of poly (ADP-ribosyl)ation in signal transduction. It has been demonstrated that PARP-deficient cells are defective in NF-κB-dependent transcriptional activation and show a down-regulation of iNOS after genotoxic stress (Hassa and Hottiger, 1999; Oliver et al., 1999). Similarly, pharma-

cological inhibitors of PARP abolish mRNA expression of iNOS, interleukin-6 and TNF-α in in vitro cultured cells (Hauschildt et al., 1997). Furthermore, we have recently demonstrated that AP-1 DNA binding is completely abolished in PARP-deficient murine fibroblasts, most probably secondary to alterations of the AP-1 dimer phosphorylation (Andreone et al., 2003). The specific mechanism of PARP activation in regulating transcription remains to be elucidated. Changes in cellular energetics after PARP activation may interfere with calcium sequestration and biosynthetic processes (Althaus and Richter, 1987). Poly (ADP-ribosyl)ation may lead to the relaxation of chromatin with the consequence that genes become more accessible to the RNA-polymerase (De Murcia et al., 1988; Meisterernst et al., 1997).

Interestingly, we noticed a slight but not significant increase of the nuclear binding of NF-κB and AP-1 after intracolonic administration of ethanol when compared to time 0. The nuclear binding of NF-κB and AP-1 exhibited similar kinetics as those observed in colon of rats receiving intracolonic administration of TNBS and was not significantly modified by treatment with the PARP inhibitors. Since ethanol is given to break the mucosal barrier and to allow penetration of TNBS into the bowel wall (Elson et al., 1995), the slight changes of NF-κB and AP-1 observed in ethanol-treated rats may reflect this initial barrier dysfunction.

Downstream targets for the transcription initiated by NF-κB and/or AP-1 are genes controlling the expression of most inflammatory mediators and immuno-modulatory, such as iNOS, chemokines and cytokines. The pathological role of iNOS in human and experimental inflammatory bowel disease has been suggested by numerous studies, including our previous findings (Boughton-Smith et al., 1993; Grisham, 1994; Zingarelli et al., 1998, 1999a). High levels of NO from activated iNOS are toxic and can damage tissue directly and/or by the formation of peroxynitrite after reaction with superoxide (Miller et al., 1995; Zingarelli et al., 1998, 1999a). In our study, treatment with the PARP inhibitors significantly attenuated the increase in plasma NO metabolites and colonic nitrotyrosine formation. Our data are in agreement with previous findings demonstrating that abrogation of PARP activity leads to down-regulation of iNOS expression, most probably to alteration of transcription (Hauschildt et al., 1997; Hassa and Hottiger, 1999; Oliver et al., 1999). In our study, a moderate production of nitrate/nitrate was still observed at 3 and 7 days after TNBS administration and it was not abolished by PARP inhibition. The maintenance of this production may suggest that a later release of NO or nitrogen derivatives may derive from the constitutive NOS or non-enzymatic sources.

Another potential mechanism by which PARP inhibition improved tissue damage in our model of colitis is the reduction of neutrophil recruitment into the site of inflammation. Accumulation and activation of inflammatory cells are some of the initial events of tissue injury and are regulated at the transcription level. For example, expression of adhesion molecules, such as P-selectin, E-selectin and intercellular adhesion molecule-1 (ICAM-1), is regulated by genes responsive to NF-κB and AP-1 (Karin et al., 1997; Baeuerle, 1998). Therefore, we may hypothesize that pharmacological inhibition of PARP may also inhibit recruitment of inflammatory cells at the transcription level. This hypothesis is supported by our previous findings demonstrating that genetic ablation of PARP completely abolished ICAM-1 and P-selectin expression in inflamed colon in the mouse (Zingarelli et al., 1999b). Furthermore, it has been demonstrated that 3-aminobenzamide reduced AP-1 binding activity to the promoter of ICAM-1 in human endothelial cells (Roebuck et al., 1995). The benzamide structure of 3-aminobenzamide has some potential for hydroxyl scavenging (Cantoni et al., 1987), which may represent an additional protective effect of 3-aminobenzamide. However, in previous studies, we have demonstrated that 3-aminobenzamide does not scavenge peroxynitrite, an important pathogenetic oxidant of colitis (Zingarelli et al., 1997). In previous experiments, we have demonstrated that in PARP-deficient mice, TNBS administration resulted in early colonic injury similar to the damage observed in wild-type mice; however, PARP-deficient mice exhibited a faster resolution (Zingarelli et al., 1999b). In our present study, we observed that treatment with pharmacological inhibition of PARP provided beneficial effects at very early time points. This difference may be explained by the fact that the TNBS-induced model of colitis differs in different species of animals. Variability of susceptibility, resistance, immunologic response and clinical course has, in fact, been reported among mice and rats (Elson et al., 1995; Fiocchi, 1998). Furthermore, it is also possible that in our rat model, 3-aminobenzamide and 1,5-dihydroxyisoquinoline may exert PARP-independent effects. Nevertheless, similar to the findings shown in this study, it appears that the protective effects of genetic inhibition of PARP may also be ascribed to alteration of the signal transduction pathway, as we have observed that amelioration of TNBS-induced colitis is associated with reduction of activation of AP-1 pathway in PARP-deficient mice (author's personal observations).

The amelioration of colon architecture observed in rats treated with the PARP inhibitors was associated with reduction of apoptotic death. It is interesting to note that after treatment with the PARP inhibitors, a scarce apoptotic process was preferentially organized in the epithelial surface and well paralleled with the earlier repair of mucosal lesions. However, the role of PARP in apoptosis remains to be determined since conflicting data have been reported. It has been proposed that preventing PARP activation increased the sensitivity of cells to apoptosis-inducing agents (Pieper et al., 1999). Nevertheless, our data show that inhibition of PARP reduces the formation of reactive oxygen and nitrogen species, thus reducing ideal triggers for the apoptotic process.

In conclusion, we demonstrated that pharmacological inhibitors of PARP exert therapeutic effects in experimental colitis. Similar to our findings, Jijon et al. (2000) have shown that pharmacological inhibition of PARP activity by 3-aminobenzamide dramatically improved barrier and metabolic function in a chronic model of colitis in mice genetically deficient of interleukin-10. According to our data, the anti-inflammatory properties of PARP inhibitors may also be ascribed, at least in part, to their ability to modulate the signal transduction mediated by NF-κB and AP-1. However, further studies are required to identify the precise molecular mechanisms of this class of drugs. Furthermore, it must be taken under consideration that TNBS-induced colitis may not represent the best experimental model to investigate the pathophysiology of human Crohn's disease or ulcerative colitis. Therefore, further investigation is needed to ascertain the potential application of PARP inhibitors and their clinical efficacy as therapeutic tools in human inflammatory bowel disease.

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