

Adelmidrol, a Palmitoylethanolamide Analogue, as a New Pharmacological Treatment for the Management of Inflammatory Bowel Disease

Marika Cordaro, Daniela Impellizzeri, Enrico Gugliandolo, Rosalba Siracusa, Rosalia Crupi, Emanuela Esposito, and Salvatore Cuzzocrea

Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy (M.C., D.I., E.G., R.S., R.C., E.E., S.C.); and Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, St. Louis, Missouri (S.C.)

Received June 20, 2016; accepted September 1, 2016

ABSTRACT

Leukocyte infiltration, improved levels of intercellular adhesion molecule 1 (ICAM-1), and oxidative stress in the colon are the principal factors in inflammatory bowel disease. The goal of the current study was to explore the effects of adelmidrol, an analog of the anti-inflammatory fatty acid amide signaling molecule palmitoylethanolamide, in mice subjected to experimental colitis. Additionally, to clarify whether the protective action of adelmidrol is dependent on the activation of peroxisome proliferator-activated receptors (PPARs), we investigated the effects of a PPAR γ antagonist, GW9662, on adelmidrol action. Adelmidrol (10 mg/kg daily, o.s.) was tested in a murine experimental model of colitis induced by intracolonic administration of dinitrobenzene sulfonic acid. Nuclear factor- κ B translocation, cyclooxygenase-2, and phosphoextracellular signal-regulated kinase, as well as tumor necrosis factor- α and interleukin-1 β , were significantly increased in colon tissues after dinitrobenzene sulfonic acid

administration. Immunohistochemical staining for ICAM-1, P-selectin, nitrotyrosine, and poly(ADP)ribose showed a positive staining in the inflamed colon. Treatment with adelmidrol decreased diarrhea, body weight loss, and myeloperoxidase activity. Adelmidrol treatment, moreover, reduced nuclear factor- κ B translocation, cyclooxygenase-2, and phosphoextracellular signal-regulated kinase expression; proinflammatory cytokine release; and the incidence of nitrotyrosine and poly(ADP)ribose in the colon. It also decreased the upregulation of ICAM-1 and P-selectin. Adelmidrol treatment produced a reduction of Bax and an intensification of Bcl-2 expression. This study clearly demonstrates that adelmidrol exerts important anti-inflammatory effects that are partly dependent on PPAR γ , suggesting that this molecule may represent a new pharmacologic approach for inflammatory bowel disease treatment.

Introduction

Ulcerative colitis (UC) and Crohn disease (CD) are the foremost categories of inflammatory bowel disease (IBD), a class of autoimmune diseases in which the body's own immune system assaults elements of the digestive apparatus (Reddy et al., 1991). In UC, the inflammatory process involves the mucosa and extends by continuity, beginning from the rectum. Two of the key characteristic symptoms of UC are bloody diarrhea and abdominal discomfort associated with fever (Ordás et al., 2012). In CD, the inflammatory event involves the entire wall of the gastrointestinal area and is propagated segmentally, not by continuity, from the oral cavity to the rectum (Baumgart and Sandborn, 2012). The most representative symptoms of CD are abdominal pain, loss of body mass, anemization, and diarrhea

associated with fever (Riordan et al., 1998). Chronic intestinal inflammation is linked with nitrosative and oxidative stress, which have been linked in numerous human diseases, including IBD (Wendland et al., 2001). Substantial evidence proposes that IBD is accompanied by an imbalance among reactive oxygen species (ROS) and antioxidant activity, which generates oxidative stress as the result of ROS overproduction such as decreased antioxidant activity (Halliwell, 1997).

Macrophages play a focal role in the improvement of non-caseous epithelioid granuloma in the intestinal mucosa, which is typical of Crohn's disease; they are involved also in the mucosal immune response. Activated macrophages produce cytokines, for example, tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , among others. Additionally, recruitment of inflammatory cells from circulation is a main process in amplifying the inflammatory response (Sandborn and Hanauer, 1999). TNF- α and IL-1 β increase the levels of adhesion molecules in the vascular endothelium, leading to penetration

This study was supported by Grant PON01-02512.
dx.doi.org/10.1124/mol.116.105668.

ABBREVIATIONS: CD, Crohn disease; COX-2, cyclooxygenase-2; DNBS, dinitrobenzene sulfonic acid; IBD, inflammatory bowel disease; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; KO, knockout; MDA, 1,1,3,3-tetramethoxypropan/99% malondialdehyde bis (dymethylacetal)/99%; MPO, myeloperoxidase; NAE, N-acelethanolamine; NF- κ B, nuclear factor- κ B; PBS, phosphate-buffered saline; PEA, palmitoylethanolamide; p-ERK, phosphoextracellular signal-regulated kinase; PPAR, peroxisome proliferator-activated receptors; ROS, reactive oxygen species; TNF, tumor necrosis factor; UC, ulcerative colitis; WT, wild type.

of inflammatory cells into the mucosal layer. Critical performers in this process are the selectins, vascular cell adhesion molecule-1, and intercellular adhesion molecule 1 (ICAM-1), which are expressed on the vascular endothelium surface (Koizumi et al., 1992).

Several animal models of IBD have been established that show similarities to human CD, although most do not predict what happens in human immunology. Amid these models, colonic inflammation provoked by the intrarectal administration of dinitrobenzene sulfonic acid (DNBS) in mice is considered a well-documented model for identification of new pharmacologic targets to treat human CD (Impellizzeri et al., 2015a). Such approaches include anti-TNF-antibodies, whose main aim is to induce clinical remission on a long-term basis and thereby effect improved patient quality of life.

Different studies have determined that N-acylethanolamines (NAEs), which are widely expressed in mammals, are involved in a range of physiologic functions, including inflammation, neurotransmission, analgesia, reproduction, appetite, and cytoprotection (Schmid and Berdyshev, 2002). Like other NAEs, palmitoylethanolamide (PEA) has been extensively studied for its anti-inflammatory and neuroprotective effects (Hansen et al., 2002; Scuderi et al., 2012). PEA inhibits mast cell activation, stabilizes mitochondrial function, and inhibits degradation of the endocannabinoid anandamide, which also has neuroprotective effects. Further, PEA may activate peroxisome proliferator-activated receptor α (PPAR α) and/or potentiate microglial cell motility in a cannabinoid receptor-independent manner (Skaper et al., 1996; Nagayama et al., 1999; Franklin et al., 2003; Impellizzeri et al., 2015b). Interestingly, as previously demonstrated, in addition to the PPAR α receptor activation, the PPAR δ and PPAR γ receptors also contribute to the anti-inflammatory activity of PEA (D'Agostino et al., 2007; Paterniti et al., 2013).

Moreover, PEA reportedly improved outcome in a murine model of experimental colitis, the effect being mediated by cannabinoid CB2 receptors GPR55 and PPAR α and modulated by transient receptor potential cation channel subfamily V member 1, all of which are involved in the control of intestinal inflammation (Borrelli et al., 2015). The anti-inflammatory ethanolamide derivative adelmidrol (international nonproprietary name) is a naturally occurring saturated dicarboxylic acid found in trace amounts in the human body and in some whole grains. Adelmidrol (*N,N'*-bis(2-hydroxyethyl)nonanediamide) possesses both hydrophilic and hydrophobic properties that favor its solubility both in aqueous and organic media (De Filippis et al., 2009). Topical treatment with adelmidrol is reported to increase mast cell granular density, suggesting a decrease in their degranulation (De Filippis et al., 2009). In addition, adelmidrol displayed some benefit in a pilot study on mild atopic dermatitis (Pulvirenti et al., 2007).

Our study was designed to evaluate the effects of adelmidrol in an animal model of IBD. To investigate whether the mechanism of action of adelmidrol treatment was similar to one previously studied for PEA, we have investigated the role of the PPAR α and PPAR γ and CB2 receptors on the adelmidrol anti-inflammatory property.

Materials and Methods

Animals. Male adult CD1 mice (25–30 g, Envigo, Italy) and male mice (2027 g, Envigo, Italy) with a targeted disruption of the PPAR α gene [PPAR α knockout (KO)] and littermate wild-type controls

[PPAR α wild-type (WT)] were placed in a controlled environment and maintained on a 12-hour light/dark cycle with food and water available ad libitum. The study was permitted by the University of Messina Review Board for the care of animals. (D.M.116192 and O.J. of E.C. L 358/1 12/18/1986).

Induction of Experimental Colitis. Colitis was made as previously described (Impellizzeri et al., 2015a). After colitis, the animals were observed for 4 days. After this period, the animals were weighed and anesthetized with chloral hydrate and the abdomen opened by a midline cut. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border, washed, weighed and processed for histologic and biochemical studies.

Experimental Groups. Mice were casually divided into the following groups (10 in each group):

1. Sham + vehicle group: Vehicle solution (saline) was given by oral administration for 4 days.
2. Sham + adelmidrol (10 mg/kg): Administered o.s. for 4 days.
3. DNBS + vehicle: Mice were injected by DNBS as described, and vehicle (saline) was given o.s. each day for 4 days, starting 60 minutes after the injection of DNBS.
4. DNBS + adelmidrol (10 mg/kg): Mice were injected by DNBS as described, and adelmidrol (10 mg/kg) was given o.s. each day, starting 60 minutes after administration of DNBS.

To better investigate whether the mechanism of action of adelmidrol is related to the activation of PPAR α and PPAR γ or CB2 receptors, we performed our studies in the following experimental groups:

1. Sham + vehicle group: Vehicle solution (saline) was given o.s. for 4 days.
2. Sham + adelmidrol (10 mg/kg): Administered o.s. for 4 days.
3. Sham + SR144528 (1 mg/kg): Administered o.s. for 4 days.
4. Sham PPAR α WT + vehicle group: Vehicle solution (saline) was given orally for 4 days.
5. Sham PPAR α KO mice + vehicle group: Vehicle solution (saline) was given o.s. for 4 days.
6. Sham PPAR α WT + adelmidrol (10 mg/kg): Administered o.s. for 4 days.
7. Sham PPAR α KO mice + adelmidrol (10 mg/kg): Administered o.s. for 4 days.
8. DNBS + vehicle: Mice were injected by DNBS as described, and vehicle (saline) was given o.s. daily for 4 days, starting 60 minutes after the injection of DNBS.
9. DNBS+ vehicle: PPAR α WT mice were injected by DNBS as described.
10. DNBS+ vehicle: PPAR α KO mice were injected by DNBS as described.
11. DNBS + adelmidrol (10 mg/kg): PPAR α WT mice were injected by DNBS as described, and adelmidrol (10 mg/kg) was given o.s. daily for 4 days, starting 60 minutes after administration of DNBS.
12. DNBS + adelmidrol (10 mg/kg): PPAR α KO mice were injected by DNBS as described, and adelmidrol (10 mg/kg) was given o.s. daily for 4 days, starting 60 minutes after administration of DNBS.
13. DNBS + GW9662 (1 mg/kg): Mice were injected by DNBS as described, but GW9662 (1 mg/kg) was given o.s. daily for 4 days, starting 60 minutes after injection of DNBS.
14. DNBS + GW9662 (1 mg/kg) + adelmidrol (10 mg/kg): Mice were injected by DNBS as described, but GW9662 (1 mg/kg) was given orally 30 minutes before adelmidrol administration.
15. DNBS + SR144528 (1 mg/kg): Mice were injected by DNBS as described, but SR144528 (1 mg/kg) was given o.s. daily, for 4 days, starting 60 minutes after the injection of DNBS.
16. DNBS + SR144528 (1 mg/kg) + adelmidrol (10 mg/kg): Mice were injected by DNBS as described, but SR144528 (1 mg/kg), was given orally 30 minutes before adelmidrol administration.

Evaluation of Colon Damage. After removal, the entire colon was gently rinsed with saline solution, opened by a longitudinal cut, and immediately observed under a microscope. Colon injury (macroscopic damage score) was evaluated and scored by two independent observers as previously described: 0, no damage; 1, restricted hyperemia without ulcers; 2, linear ulcers with no important 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 5–8, one point is added for each centimeter of ulceration beyond an initial 2 cm (Zingarelli et al., 1993; Cuzzocrea et al., 2003).

Tissue Processing and Histology. Concisely, paraffin tissue slices (thickness, 7 μ m) were deparaffinized with xylene, stained with H&E and observed by light microscopy (AxioVision, Zeiss, Milan, Italy) by a qualified histopathologist. The degree of inflammation on microscopic cross-sections of the colon was graded semiquantitatively from 0 to 4 as previously described by Impellizzeri et al. (2015a), in particular, the following morphologic 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 the presence of neutrophil infiltrate in the submucosa; score 4 (highly severe), widespread necrosis with massive neutrophil infiltrate and hemorrhage (Cuzzocrea et al., 2004). The scores from all sections of each colon were averaged to give a final score for each mouse. All histologic analyses were performed in a blinded fashion.

Myeloperoxidase Assay. Neutrophil infiltration in the colon was examined by determining tissue myeloperoxidase (MPO) activity using a spectrophotometric assay with tetramethylbenzidine as substrate, according to a previously published method (Mullane et al., 1985). After DNBS injection, colon tissues were collected and weighed. Every piece of tissue was homogenized in a mixture containing 0.5% hexadecyltrimethyl ammonium bromide dissolved in 10 mM potassium phosphate buffer, pH 7, and centrifuged for 30 minutes at 20,000g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H₂O₂. The degree of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was described as the quantity of enzyme degrading 1 mmol of peroxide per minute at 37°C and expressed in U/g wet tissue.

Thiobarbituric Acid–Reactant Substances Measurement. Thiobarbituric acid–reactant substances measurement was determined in colon tissue 4 days after DNBS administration as a marker of lipid peroxidation. Thiobarbituric acid–reactant substances were calculated by comparison the OD₅₃₂ to a standard mixture of 1,1,3,3-tetramethoxypropan/99% malondialdehyde bis (dymethylacetal)/99% (MDA) (Sigma, Milan, Italy). The absorbance of the supernatant was measured spectrophotometrically at 532 nm.

Immunohistochemical Localization of TNF- α , IL-1 β , ICAM-1, P-Selectin, Poly(ADP-ribose) (PAR), and Nitrotyrosine. Seven

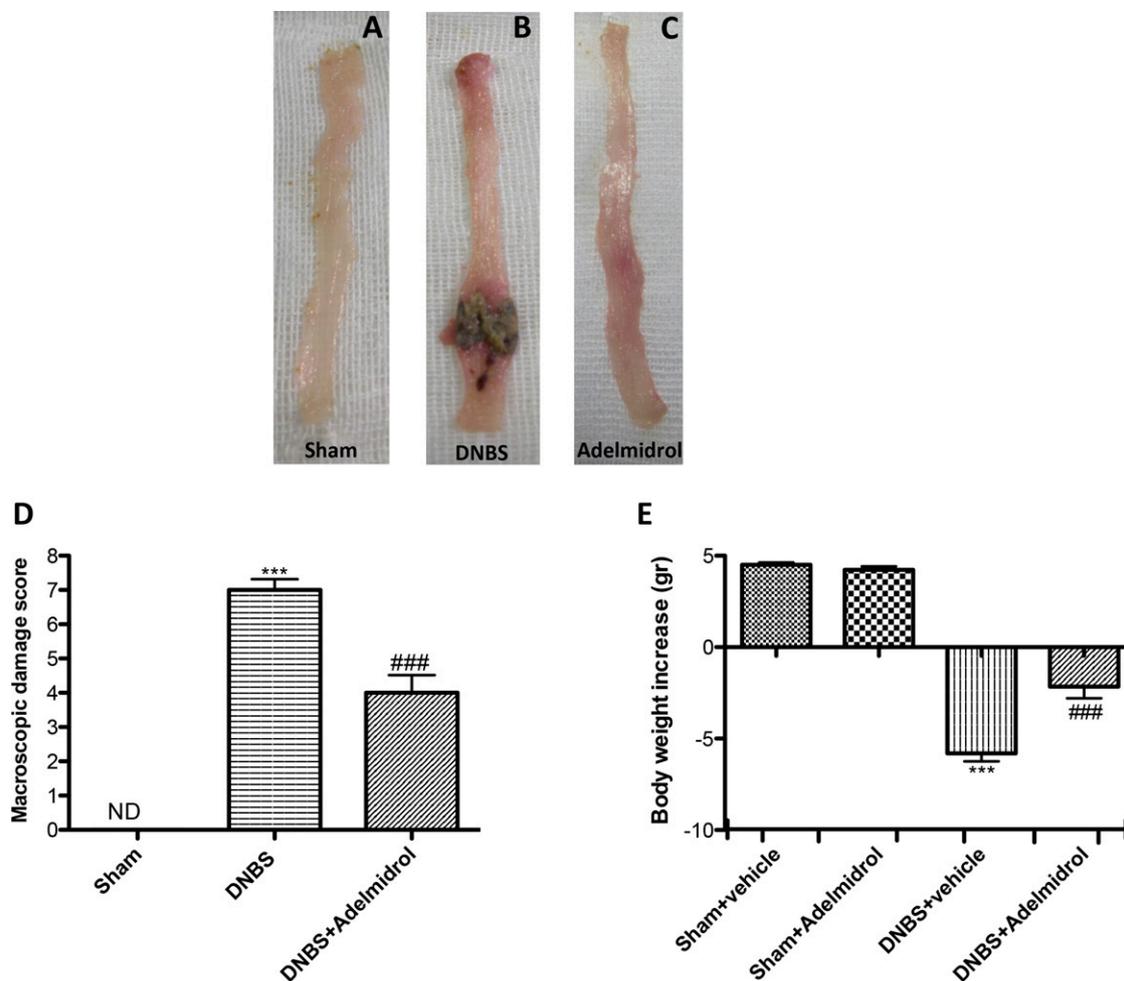


Fig. 1. Effects of adelmidrol on macroscopic damage and body weight in DNBS-induced colitis. No macroscopic alteration was observed in colon tissue from sham-treated mice (A); see macroscopic score (D). Macroscopic inspection of cecum, colon, and rectum evidenced mucosal congestion, erosion, and hemorrhagic ulcerations (B); see macroscopic score (D). Adelmidrol (10 mg/kg, o.s.) reduced significantly the extent and severity of this macroscopic damage (C); see macroscopic score (D). Moreover, adelmidrol treatment reduced the loss of body weight (E). *** $P < 0.001$ versus sham group; ### $P < 0.001$ versus DNBS group.

days after DNBS administration, colon tissues were collected and fixed for 24 hours in paraformaldehyde [4% in 0.1M phosphate-buffered saline (PBS)] at room temperature, dehydrated through a graded series of ethanol and xylene, and embedded in BioPlast Plus (Bio Optica, Milan, Italy). Thereafter, 7 μ m sections were cut from the paraffin-embedded tissue. After deparaffinization with xylene and graded ethanol as described herein, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 minutes. Slices were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 minutes. Nonspecific adsorption was diminished by incubating the section in 2% (v/v) normal goat serum in PBS for 20 minutes. Endogenous biotin and avidin binding sites were blocked by progressive incubation for 15 minutes with biotin and avidin (Vector Laboratories, Burlingame, CA), respectively. Subsequently, slices were incubated overnight with anti-TNF- α murine polyclonal antibody (1/100 in PBS, v/v; Santa Cruz Biotechnology, Dallas, Texas) or anti-IL-1 β murine polyclonal antibody (1/100 in PBS; v/v, Santa Cruz Biotechnology), anti-ICAM-1 murine polyclonal antibody (1/100 in PBS; v/v, Santa Cruz Biotechnology), anti-P-selectin murine polyclonal antibody (1/100 in PBS; v/v, Santa Cruz Biotechnology), anti-PAR murine polyclonal antibody (1/100 in PBS, v/v; Santa Cruz Biotechnology), and anti-nitrotyrosine rabbit polyclonal antibody (1:200 in PBS, v/v; Millipore, Billerica, MA). Sections were rinsed with PBS and incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG

(1:2,000; Jackson Immuno Research, West Grove, PA). Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG or biotin-conjugated goat anti-mouse IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA). To verify the binding specificity for TNF- α , IL-1 β , ICAM-1, P-selectin, PAR, and nitrotyrosine, control slices were incubated with only primary antibody or secondary antibody. In these controls, no positive staining was detected. Immunohistochemical images were collected using a Zeiss microscope and AxioVision software (Zeiss). For graphic display of densitometric analyses, the intensity of positive staining (brown staining) was measured by computer-assisted color image analysis (Leica QWin V3, UK). The percentage area of immunoreactivity (determined by the number of positive pixels) was expressed as percentage of total tissue area (red staining). Replicates for every experimental condition and histochemical staining were acquired from each mouse in all experimental groups. In sham-operated mice, the central areas of equivalent tissue sections were taken as reference points, and a comparable number of optical fields were counted (Shea, 1994). All histologic analyses were carried out by an observer without knowledge of the treatments.

Western Blot Analysis for I κ B- α , Nuclear Factor- κ B, Cyclooxygenase-2, Phosphoextracellular Signal-Regulated Kinase, Bcl-2, Bax, Lamin a/c, and β -Actin. The levels of I κ B- α , nuclear factor- κ B (NF- κ B), cyclooxygenase-2 (COX-2), phosphoextracellular signal-regulated kinase (p-ERK), Bcl-2, Bax, lamin a/c, and β -actin were calculated, as previously described, in cytosolic and

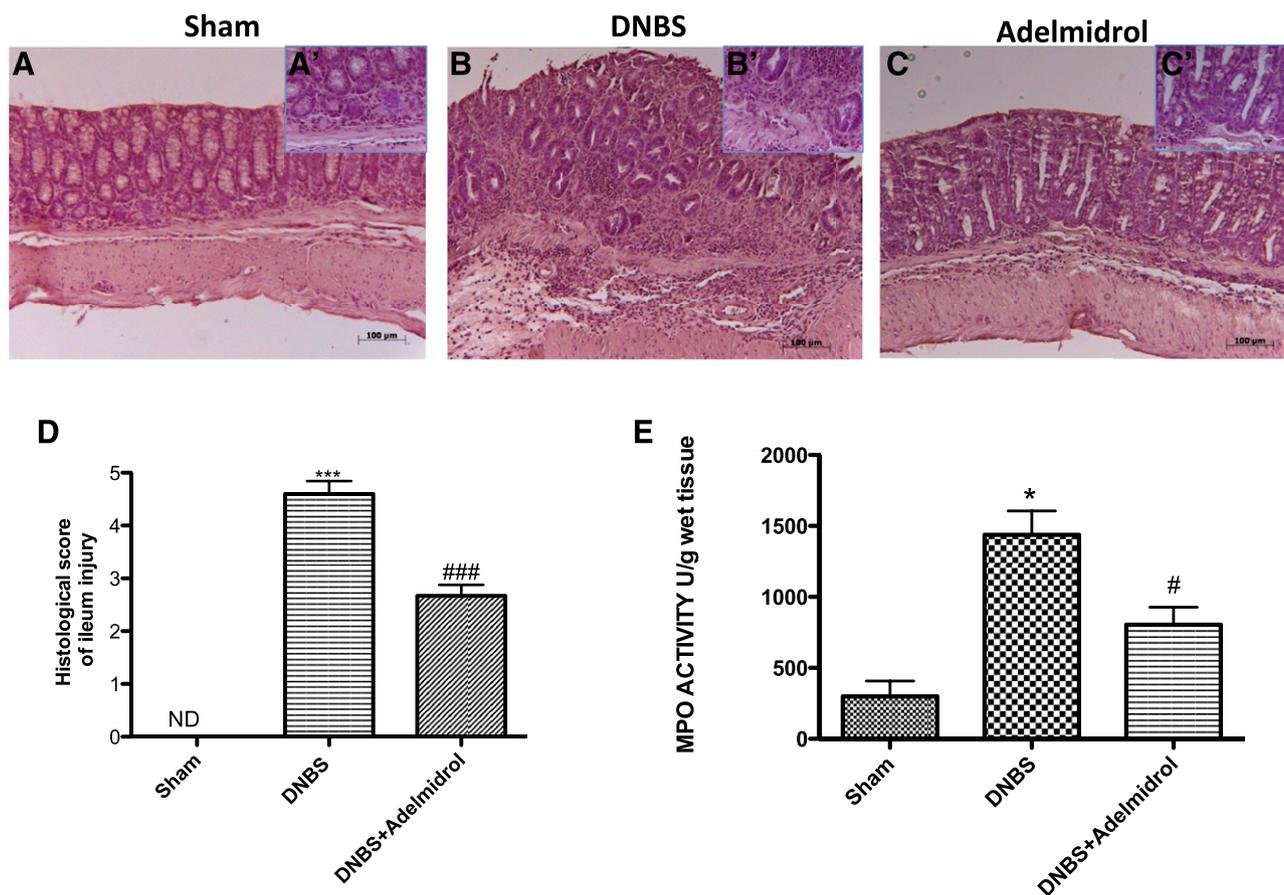


Fig. 2. Effects of adelmidrol on histologic damage and MPO activity in DNBS-induced colitis. Histologic alteration was absent in colon tissue from sham-treated mice (A,A'); histologic score (D). Four days after intracolonic administration of DNBS, the colon showed transmural necrosis and edema with a diffuse leukocyte cellular infiltrate in the submucosa (B,B'); histologic score (D). Adelmidrol (10 mg/kg, o.s.) reduced significantly the histologic signs of colon injury (C,C'); histologic score (D). Myeloperoxidase (MPO) activity was increased in DNBS-injected mice in comparison with sham-treated mice (C), and treatment with adelmidrol (10 mg/kg, o.s.) reduced the extent of PMN infiltration (determined as decrease in MPO activity) in inflamed colon (2E). * $P < 0.05$ versus sham group; # $P < 0.05$ versus DNBS group; *** $P < 0.001$ versus sham group; ### $P < 0.001$ versus DNBS group.

nuclear fractions from colon tissue collected at the end of the experiment with minor modifications (Bethua et al., 1998). Colon tissue from each mouse was suspended in extraction buffer A containing 0.2 mM phenylmethylsulfonyl fluoride, 0.15 mM pepstatin A, 20 mM leupeptin, 1 mM sodium orthovanadate, homogenized at the maximum setting for 2 minutes, and centrifuged at 12,000g \times rpm for 4 minutes at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM leupeptin, 0.2 mM sodium orthovanadate. After centrifugation 10 minutes at 12,000 rpm at 4°C, the supernatants containing the nuclear protein were stored at -80°C for further analysis. The filters were blocked with 1 \times PBS, 5% (w/v) nonfat dried milk for 40 minutes at room temperature, and successively probed with anti-I κ B- α (1/500 in PBS, v/v, Santa Cruz Biotechnology), anti-NF- κ B (1/500 in PBS, v/v, Santa Cruz Biotechnology), anti-COX-2 (1/500 in PBS, v/v, Cayman), anti-p-ERK (1/500 in PBS, v/v, Santa Cruz Biotechnology), anti-Bax (Santa Cruz Biotechnology 1/500 in PBS, v/v), anti-Bcl-2 (1/500 in PBS, v/v, Santa Cruz Biotechnology), and anti-lamin a/c (1/500 in PBS, v/v,

Santa Cruz Biotechnology) in 1 \times PBS, 5% (w/v) nonfat dried milk, 0.1% Tween-20 (PMT) at 4°C overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson Immuno Research, West Grove, PA) for 1 hour at room temperature. To establish that blots were loaded with equivalent amounts of protein lysates, they were similarly incubated with antibody against β -actin (1/1000 in PBS, v/v, Santa Cruz Biotechnology v). Relative expression of the protein bands for I κ B- α (37 kDa), NF- κ B (65 kDa), COX-2 (72 kDa), p-ERK (46 kDa), Bcl-2 (29 kDa), Bax (23 kDa), lamin a/c (65 kDa), and β -actin (42 kDa) were detected with the enhanced chemiluminescence detection system according to the manufacturer's instructions (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA). Expression of protein bands was calculated by densitometry with Bio-Rad ChemiDoc XRS + software (Hercules, CA) and standardized to β -actin levels. Images of blot signals (8-bit/600-dpi resolution) were imported to an analysis program (Image Quant TL, v2003). Commercially available molecular weight markers (10–250 kDa) were used to establish molecular weight positions.

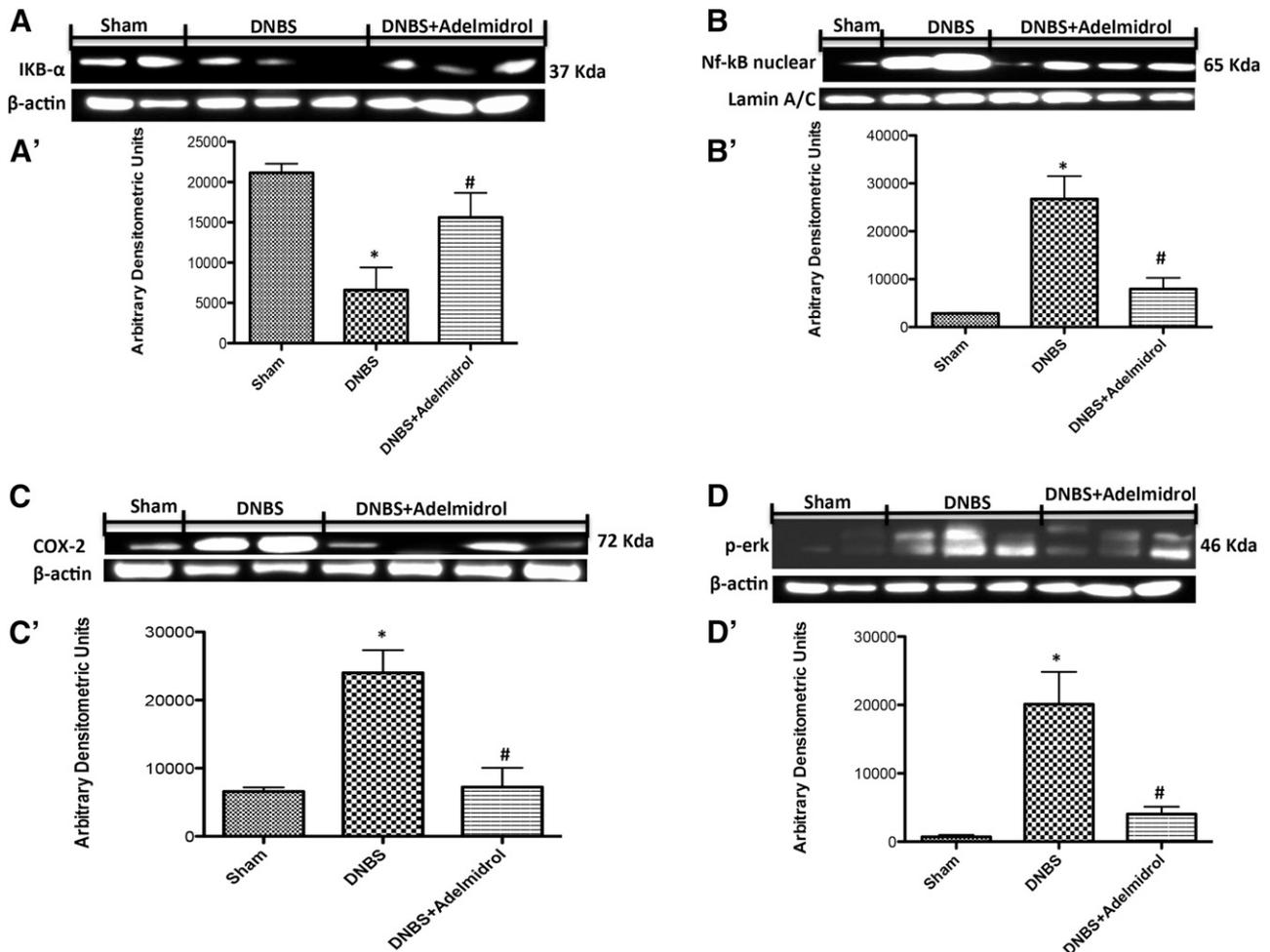


Fig. 3. Effects of adelmidrol treatment on NF- κ B pathway, COX-2, and p-ERK expression in DNBS-induced colitis. Basal expression of I κ B- α was detected in colon samples from sham-treated animals were substantially reduced in colon tissues from vehicle-treated animals 4 days after DNBS injection (A); densitometric examination (A'). Adelmidrol treatment prevented DNBS-induced I κ B- α degradation (A); densitometric examination (A'). NF- κ B p65 levels in the colon nuclear fractions were substantially increased 4 days after DNBS injection compared with sham-treated mice (B); see densitometric examination (B'). Treatment with adelmidrol (10 mg/kg, o.s.) reduced the levels of NF- κ B p65 (B); see densitometric examination (B'). Moreover, COX-2 levels were significantly increased in the colon tissue of DNBS-injected mice compared with sham mice. Treatment with adelmidrol (10 mg/kg, o.s.) significantly reduced DNBS-mediated COX-2 expression (C); see densitometric examination (C'). In addition, pERK1/2 levels were significantly increased in the colon tissues of DNBS-injected mice (D); see densitometric examination (D') compared with sham-treated mice. Adelmidrol significantly reduced the level of pERK1/2 activation (D); see densitometric examination (D'). The densitometric expression of protein bands was normalized to β -actin and lamin a/c levels. * P < 0.05 versus sham; # P < 0.05 versus DNBS.

Materials. Adelmidrol was obtained from Epitech Group Spa (Saccolongo, Italy). All compounds were purchased from Sigma Aldrich (Milan, Italy). All chemicals were of the maximum commercial grade available. All stock mixes were made in non-pyrogenic saline (0.9% NaCl; Baxter, UK).

Statistical Evaluation. All values in the images and text are expressed as mean \pm S.E.M. of n observations. For in vivo studies, n represents the number of animals. In experiments involving histology and immunohistochemistry, the pictures shown are demonstrative of at least three independent experiments. Moreover, Western blot images are representative of three different gels obtained by dividing the number of samples from 10 animals for each experimental group on different days. A P value of less than 0.05 was appraised as significant. The results were analyzed by one-way analysis of variance, followed by a Bonferroni post hoc test for multiple comparisons.

Results

Effects of Adelmidrol Treatment on the Degree of Colitis in DNBS-Challenged Mice. Histologic analysis of colonic mucosa of sham-treated mice showed an intact epithelium (Fig. 1A, see macroscopic score, Fig. 1D; Fig. 2A, A'; view histologic score, Fig. 2D). Four days after intracolonic administration of DNBS, the colon appeared

flaccid and filled with liquid stool. Macroscopic examination of cecum, colon, and rectum showed mucosal congestion, erosion, and hemorrhagic ulcerations (Fig. 1B; see macroscopic score, Fig. 1D). The histopathological features included transmural necrosis and edema and a diffuse leukocyte cellular infiltrate in the submucosa of colon sections from DNBS-injected mice (Fig. 2B, B'; view histologic score Fig. 2D). Adelmidrol (10 mg/kg, o.s.) reduced significantly the degree and severity of the macroscopic (Fig. 1C; see macroscopic score, Fig. 1D) and histologic signs of colon injury (Fig. 2C, C'; view histologic score, Fig. 2D). Moreover, 4 days after colitis induced by DNBS treatment, all mice had diarrhea and a reduction in body weight (compared with the sham groups) (Fig. 1E). Adelmidrol (10 mg/kg, o.s.) treatment significantly reduced the loss of body weight (Fig. 1E). The IBD induced by DNBS intrarectally administered was also characterized by an augmentation in MPO activity, an indicator of neutrophil accumulating in the colon (Fig. 2E). This was consistent with light microscopic observations showing the colon of vehicle-treated DNBS mice to contain a large number of neutrophils. In contrast, adelmidrol (10 mg/kg, o.s.) significantly reduced the degree of polymorphonuclear cell infiltration (determined as reduction in MPO activity) in inflamed colon (Fig. 2E).

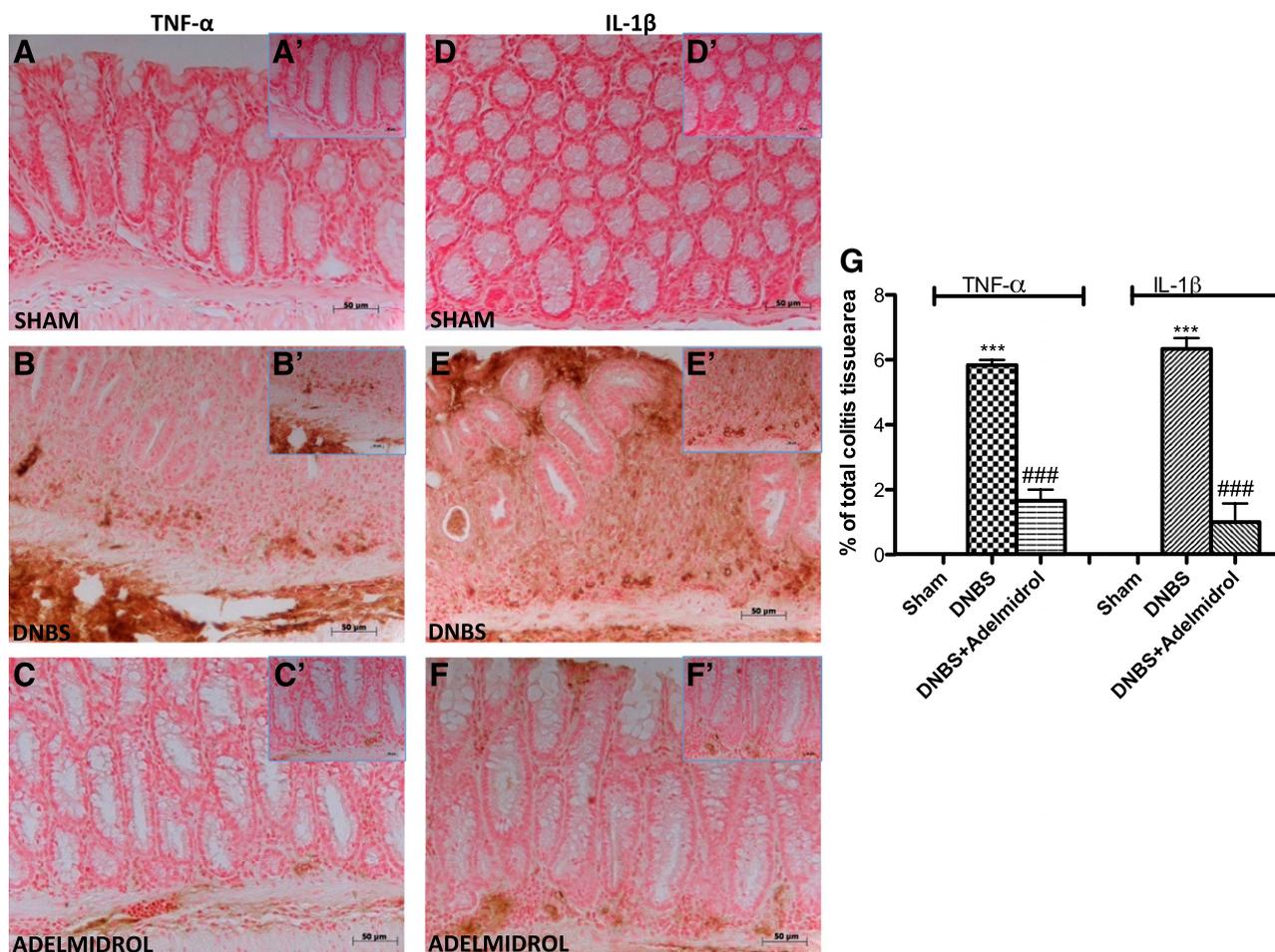


Fig. 4. Adelmidrol treatment reduces cytokine production in DNBS-induced colitis. The substantial increase in TNF- α and IL-1 β formation observed in colon samples collected from DNBS-injected mice compared with sham mice (B,E); see densitometric examination (G') was significantly attenuated in mice treated with adelmidrol (10 mg/kg, o.s.) (C,F); see densitometric examination (G'). TNF- α and IL-1 β immunostaining was not detected in colon from sham mice (A and D); see densitometric examination (G). *** P < 0.001 versus sham group; ### P < 0.001 versus DNBS group:

Effects of Adelmidrol Treatment on NF- κ B Pathway, COX-2, and p-ERK Expression in DNBS-Challenged Mice. To explore the cellular mechanism(s) underlying adelmidrol mitigation of the development of DNBS-induced colitis, we used Western blot analysis to evaluate I κ B- α degradation and nuclear NF- κ B p65 translocation. NF- κ B p65 levels in the colon nuclear fractions were substantially improved 4 days after DNBS injection compared with sham mice (Fig. 3B; see densitometric examination, Fig. 3B'). Treatment with adelmidrol (10 mg/kg, o.s.) reduced the colon levels of NF- κ B p65 (Fig. 3B; see densitometric examination Fig. 3B'). Basal expression of I κ B- α detected in colon samples from sham-treated animals were significantly reduced in colon tissues from vehicle-treated animals 4 days after DNBS injection (Fig. 3A; see densitometric examination, Fig. 3A'). Adelmidrol treatment prevented DNBS-induced I κ B- α degradation (Fig. 3A; see densitometric examination, Fig. 3A'). In addition, phosphorylation of ERK1/2 plays a role in the control of gene expression, for example, COX-2, mediating the inflammatory responses. Basal levels of COX-2 in homogenates from colon tissues of sham-operated animals (Fig. 3C; see densitometric examination, Fig. 3C') were significantly increased in colon tissue of

DNBS-injected mice. Treatment of mice with adelmidrol (10 mg/kg, o.s.) reduced DNBS-mediated COX-2 expression (Fig. 3C; see densitometric examination, Fig. 3C'). This increase in COX-2 expression appeared to correspond to an activation of signal transduction pathways involved in its regulation, as pERK1/2 levels were significantly increased in the colon tissues of DNBS-injected mice (Fig. 3D; see densitometric examination, Fig. 3D'). Likewise, treatment with adelmidrol significantly reduced pERK1/2 activation (Fig. 3D; see densitometric examination, Fig. 3D').

Adelmidrol Treatment Reduces Cytokine Production in DNBS-Challenged Mice. Immunohistological analysis of colon tissue was carried out to determine whether adelmidrol might modulate the secondary inflammatory reaction also by regulating secretion of cytokines, such as TNF- α and IL-1 β . A substantial increase of TNF- α and IL-1 β production was found in colon samples collected from DNBS-injected mice compared with sham-operated mice (Fig. 4, B and E; see densitometric examination 4G). Colon levels of TNF- α and IL-1 β were significantly attenuated in mice treated with adelmidrol (10 mg/kg, o.s.) (Fig. 4, C and F; see densitometric examination, Fig. 4G). There was no

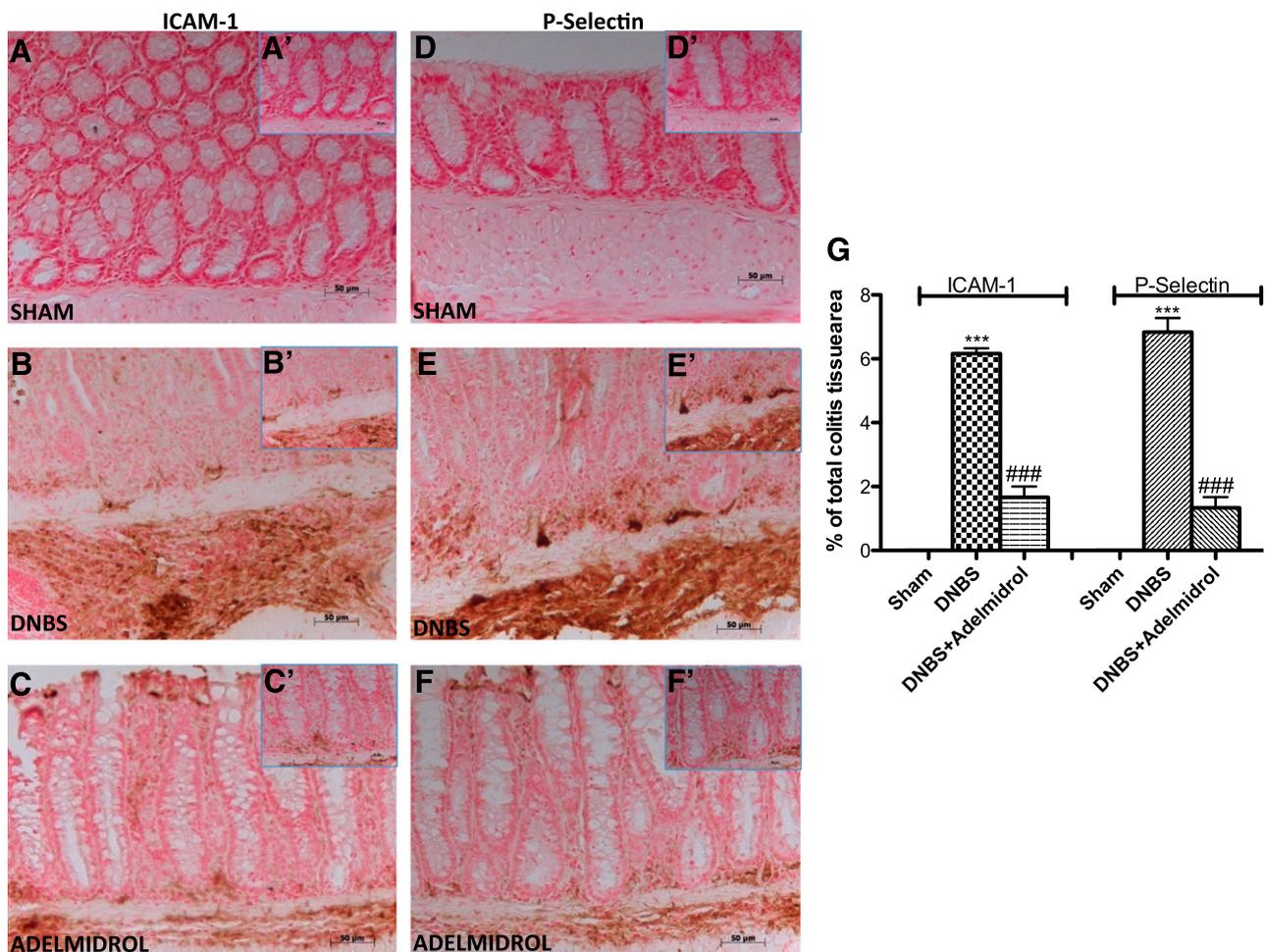


Fig. 5. Adelmidrol treatment reduces ICAM-1 and P-selectin expression in DNBS-induced ICAM-1. P-selectin immunoreactivity (B and E); [see densitometric examination (G)] was substantially increased in vessels of the lamina propria and submucosa as well as in epithelial cells of injured colon and in infiltrated inflammatory cells in tissues from DNBS-injected mice. Treatment with adelmidrol (10 mg/kg, o.s.) reduced ICAM-1 and P-selectin immunostaining (C and F); see densitometric examination (G) in the colon tissues from DNBS-injected mice. No positive staining for ICAM-1 and P-selectin was observed in colon tissues from sham-treated mice (A and D; see densitometric examination (G)). *** $P < 0.001$ versus sham group; ### $P < 0.001$ versus DNBS group.

staining for TNF- α and IL-1 β in colon obtained from sham groups of mice (Fig. 4, A and D; see densitometric examination, Fig. 4G).

Adelmidrol Treatment Reduces ICAM-1 and P-Selectin Expression in DNBS-Challenged Mice. The potential contribution of intestinal expression of ICAM-1 and P-selectin to cell recruitment throughout colon inflammation was next examined. Positive staining for ICAM-1 and P-selectin (Fig. 5, B and E; see densitometric examination, Fig. 5G) was significantly increased in vessels of the lamina propria and submucosa as well as in epithelial cells of injured colon and in infiltrating inflammatory cells in damaged tissues from DNBS-injected mice. Treatment with adelmidrol (10 mg/kg, o.s.) reduced staining for ICAM-1 and P-selectin (Fig. 5, C and F; see densitometric examination, Fig. 5G) in colon tissues of DNBS-injected mice. No positive staining for ICAM-1 and P-selectin was observed in colon tissues of sham-treated mice (Fig. 5, A and D; see densitometric examination, Fig. 5G).

Effects of Adelmidrol Treatment on Nitrotyrosine and PAR Formation and Lipid Peroxidation in DNBS-Challenged Mice. Immunohistochemistry for PAR revealed positive staining localized in the nuclei of inflammatory cells in colon tissues from DNBS-injected mice (Fig. 6B; see densitometric examination, Fig. 6G). Adelmidrol (10 mg/kg, o.s.) significantly reduced the extent of PAR immunoreactivity in the colon (Fig. 6C; see densitometric examination, Fig. 6G). Four days after DNBS administration, nitrotyrosine, an indicator of nitrosative stress, was assessed by immunohistochemical analysis in terminal colon sections to identify “peroxynitrite formation” and/or other nitrogen derivatives formed during experimental colitis. Colon sections from sham-treated mice did not stain for nitrotyrosine (Fig. 6D; see densitometric examination, Fig. 6G), but sections from DNBS-injected mice displayed positive nitrotyrosine immunostaining, mainly in inflammatory cells and around vessels (Fig. 6E; see densitometric examination, Fig. 6G). Treatment with adelmidrol (10 mg/kg, o.s.) reduced the degree of nitrotyrosine

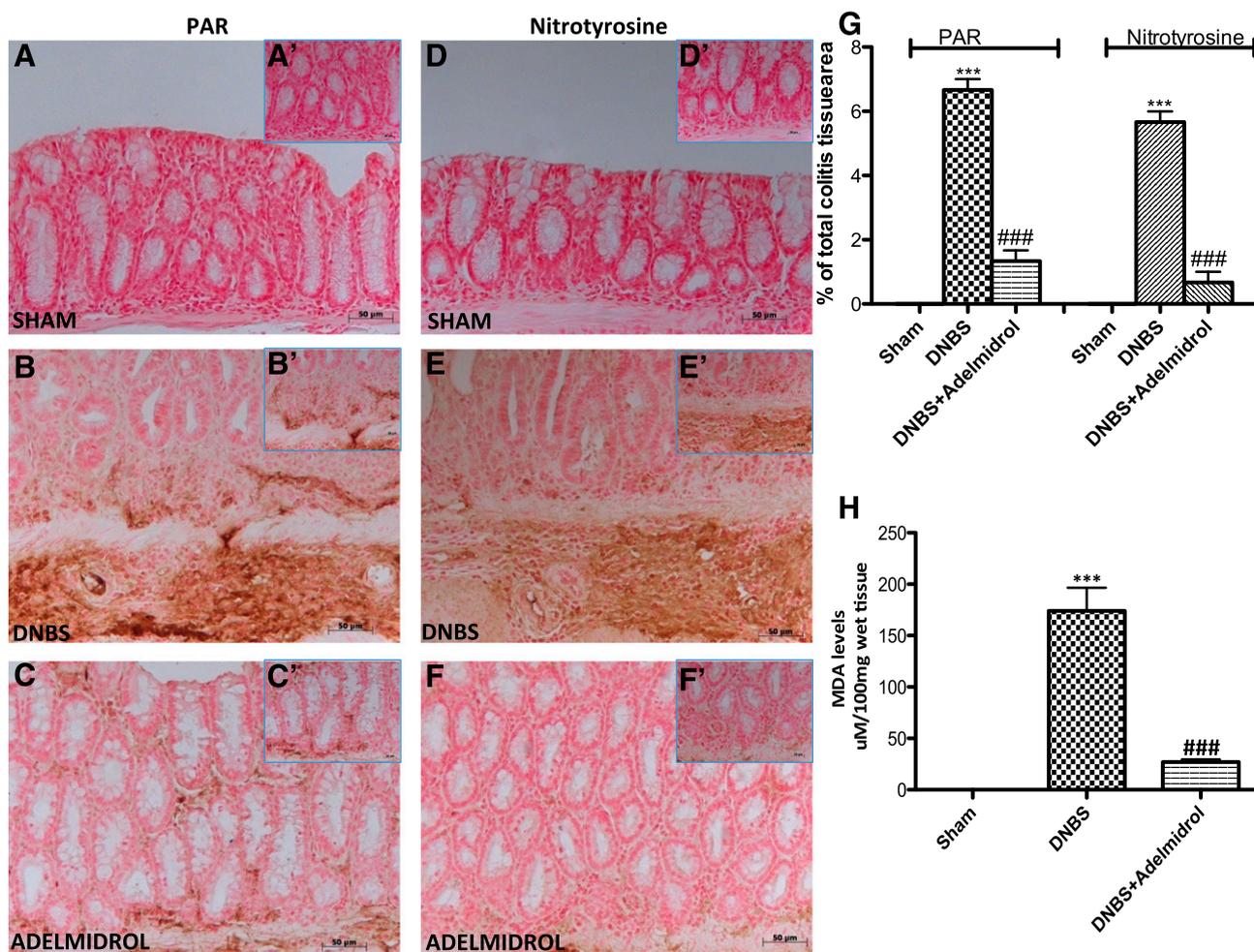


Fig. 6. Effects of adelmidrol treatment on nitrotyrosine and PAR formation and lipid peroxidation in DNBS-induced colitis. PAR immunoreactivity was visible in the nuclei of inflammatory cells in colon tissues from DNBS-injected mice compared with sham-treated animals (B); see densitometric examination (G). Adelmidrol (10 mg/kg, o.s.) significantly reduced the extent of immunostaining for PAR in the colon (C); see densitometric examination (G). Whereas colon sections from sham-treated mice did not stain for nitrotyrosine (D); see densitometric examination (G), sections from DNBS-injected mice exhibited positive immunostaining for nitrotyrosine (E); see densitometric examination (G). Treatment with adelmidrol (10 mg/kg, o.s.) reduced the degree of positive immunostaining for nitrotyrosine (F); see densitometric examination (G) in the colon. In addition, plasma levels of thiobarbituric acid-reactant substances (an indicator of lipid peroxidation) were significantly increased (H) in plasma collected from mice subjected to DNBS compared with sham-treated animals. Treatment with adelmidrol (10 mg/kg, o.s.) markedly attenuated the DNBS-induced rise in thiobarbituric acid-reactant substances (H). *** $P < 0.001$ versus sham group; ### $P < 0.001$ versus DNBS group.

immunoreactivity in the colon (Fig. 6F; see densitometric examination, Fig. 6G). In addition, plasma levels of thiobarbituric acid–reactant substances, as an indicator of lipid peroxidation, increased significantly in mice subjected to DNBS compared with sham-operated mice (Fig. 6H). Thiobarbituric acid–reactant substance levels were significantly attenuated in DNBS treated with adelmidrol (10 mg/kg, o.s.) (Fig. 6H).

Effects of Adelmidrol Treatment on the Apoptotic Pathway in DNBS-Challenged Mice. To test whether colon damage was connected with apoptosis, 4 days after DNBS, the presence of proteic effectors of canonical mitochondrial apoptosis, such as Bax and Bcl-2, was investigated by Western blot analysis. Bax levels were appreciably increased in the colon of mice subjected to DNBS (Fig. 7B; see densitometric examination, Fig. 7B'). In contrast, adelmidrol treatment (10 mg/kg, o.s.) prevented DNBS-induced Bax expression (Fig. 7B; see densitometric examination, Fig. 7B'). On the other hand, adelmidrol administration (10 mg/kg, o.s.) increased Bcl-2 expression in DNBS-injected mice (Fig. 7A; see densitometric examination, Fig. 7A'), essentially reaching the basal level of Bcl-2 (Fig. 7A; see densitometric examination, Fig. 7A').

Role of PPAR γ Receptors in the Systemic Anti-inflammatory Effects of Adelmidrol in DNBS-Induced Colitis. To investigate the mechanism of adelmidrol action, we used the potent PPAR γ antagonist GW9662 in the model of DNBS-induced colitis. Histologic evaluation by H&E staining revealed that mice that received GW9662 after DNBS injection showed an intensification of the damage as well as an increase in MPO levels, similar to the DNBS group (Fig. 8, B and C; see macroscopic score, Fig. 8F; Fig. 9, B and C; view histologic score, Fig. 9F). Moreover, DNBS-injected mice pretreated with GW9662 displayed limited protection by adelmidrol, suggesting a possible antagonist effect of GW9662 pretreatment on adelmidrol action (Fig. 8, D and E; see macroscopic score, Fig. 8F; Fig. 9, D and E; view histologic

score, Fig. 9F). Additionally, 4 days after colitis induced by DNBS treatment, mice pretreated with GW9662 showed a major loss in body weight (Fig. 8G) and intensification of MPO activity (Fig. 9G) compared with adelmidrol-treated mice.

Role of PPAR α and CB2 Receptors in the Systemic Anti-inflammatory Effects of Adelmidrol in DNBS-Induced Colitis. Histologic evaluation by H&E staining showed a transmural necrosis and edema and a diffuse leukocyte cellular infiltrate in the submucosa of colon sections from DNBS groups (data not shown). The histologic alteration was also found in PPAR α WT and KO mice and in DNBS-injected mice that have received SR144528 (Fig. 10, A, A', C, C', E, and E'; view histologic score, Fig. 10, G and H) compared with sham groups (data not shown). Moreover, adelmidrol treatment was able to decrease the histologic alteration in all experimental groups, suggesting that the absence of PPAR α receptor or pretreatment with SR144528 did not modify the action of adelmidrol (Fig. 10, B, B', D, D', F, and F'; view histologic score, Fig. 10, G and H).

Discussion

IBD is a chronic, relapsing, inflammatory condition of the gastrointestinal tract and includes UC and CD, which show differences in pathology and clinical characteristics. Presently, the causes and pathogenesis of IBD are poorly understood. It is widely accepted that the pathogenesis of IBD involves genetic and environmental factors (Pierik et al., 2005; Ye et al., 2015). Until recently, the main goal of IBD therapy was to achieve and preserve clinical remission, whereas today's therapeutic aim is that of a prolonged remission characterized by sustained clinical remission, complete mucosal healing, and normalization of serologic markers of inflammation. The most frequently applied biologic agents in CD and UC are TNF- α -targeted therapies, a class of medication that has modernized the treatment of these

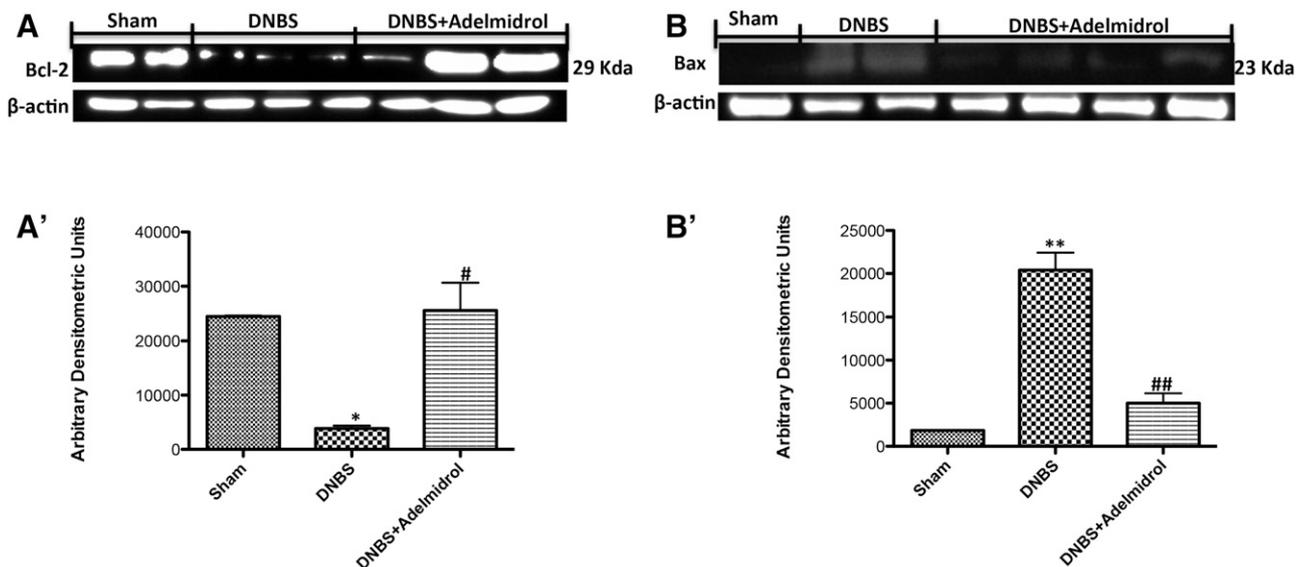


Fig. 7. Effects of adelmidrol treatment on apoptotic pathway in DNBS-induced colitis. Basal Bcl-2 levels in sham mice were markedly diminished after DNBS injection (A); densitometric examination (A'), an effect overcome with adelmidrol administration (10 mg/kg, o.s.) (A); see densitometric examination (A'). Bax levels appreciably increased in colon tissue from mice subjected to DNBS and were prevented from doing so in adelmidrol-treated mice (10 mg/kg, o.s.) (B); see densitometric examination (B'). Densitometric analysis of protein bands was normalized to the level of β -actin. * $P < 0.05$ versus sham; # $P < 0.05$ versus DNBS; ** $P < 0.01$ versus sham; ## $P < 0.01$ versus DNBS.

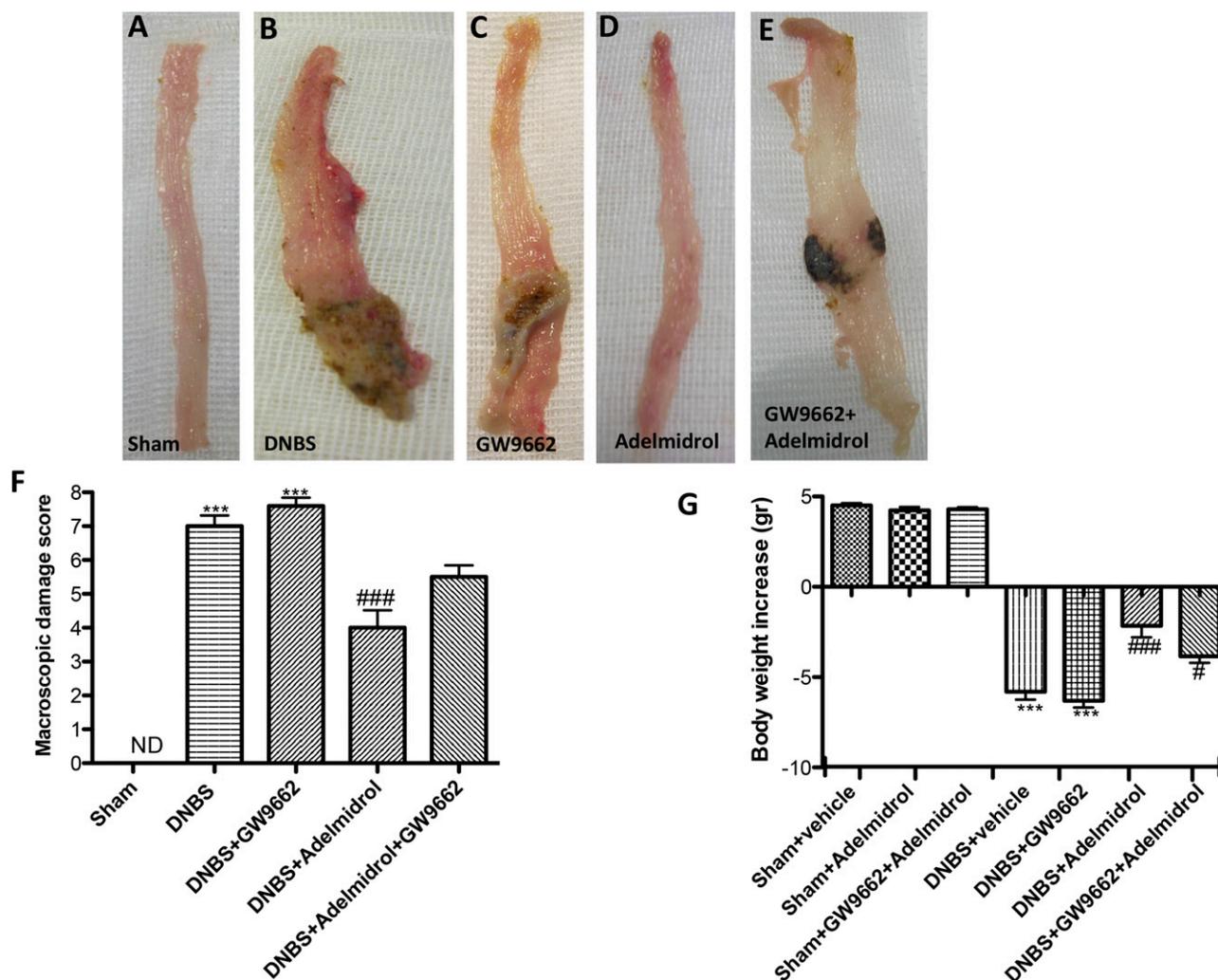


Fig. 8. Role of PPAR γ receptors in the systemic anti-inflammatory effects of adelmidrol in DNBS-induced colitis. Macroscopic inspection of cecum, colon, and rectum evidenced mucosal congestion, erosion, and hemorrhagic ulcerations (B); macroscopic score (F) compared with sham (A); macroscopic score (F). GW9662 administration did not show a significant difference compared with DNBS group (C); see macroscopic score (F). Adelmidrol (10 mg/kg, o.s.) significantly reduced the extent and severity of this macroscopic damage (D); see macroscopic score (F). Moreover, adelmidrol treatment reduced also the loss of body weight (G). DNBS-injected mice pretreated with GW9662 did not show a reduction of macroscopic damage (E); see macroscopic score (F). Moreover, mice pretreated with GW9662 showed a major loss in body weight (G) compared with adelmidrol-treated mice. *** $P < 0.001$ versus sham group; # $P < 0.05$ versus DNBS group; ### $P < 0.001$ versus DNBS group.

disorders and the quality of life for patients; however, they also pose risks of developing various side effects, including infections, exacerbation of some neurologic manifestations, cutaneous lesion, or antibody production (Casili et al., 2016).

Adelmidrol, a diethanolamide derivative of azelaic acid, is effective in the treatment of human inflammatory disorders, and its mechanism of action has been extensively investigated (Mastrofrancesco et al., 2010). Adelmidrol belongs to the aliamide family, with anti-inflammatory and antinociceptive properties similar to those of PEA and able to control mast cell hyper-reactivity in several pathophysiological conditions (Aloe et al., 1993; Costa et al., 2008; Genovese et al., 2008; De Filippis et al., 2011; Esposito et al., 2011).

Here, we investigated the beneficial effects of adelmidrol in an experimental model of IBD. In particular, adelmidrol attenuated DNBS-induced colitis in mice. At the macroscopic and histologic levels, epithelial disruption was significantly reduced in mice treated with adelmidrol. Further, mice treated with adelmidrol were more resistant to DNBS-induced

colitis, showing a marked resolution of the macroscopic and histologic markers of the inflammatory process. This was also reflected in a decreased weight loss as well as a reduction of MPO activity in mice treated with adelmidrol compared with the DNBS-injected group.

NF- κ B is an important mediator of inflammation. In response to a wide range of stimuli, including oxidative stress, infection, hypoxia, extracellular signals, and inflammation, I κ B α is phosphorylated by I κ B kinase (Bowie and O'Neill, 2000). This result is the release of the NF- κ B dimer, which then translocates to the nucleus. DNBS induced a significant degradation of I κ B α and a consequent increase in the nuclear translocation of the subunit p65, whereas adelmidrol treatment significantly reduced NF- κ B translocation and inhibited I κ B α degradation. Moreover, intestinal epithelial cells express COX-2 when stimulated by proinflammatory factors, such as lipopolysaccharide and oxidative stress (Longo et al., 1998). Adelmidrol reduced COX-2 and p-ERK DNBS-induced expression.

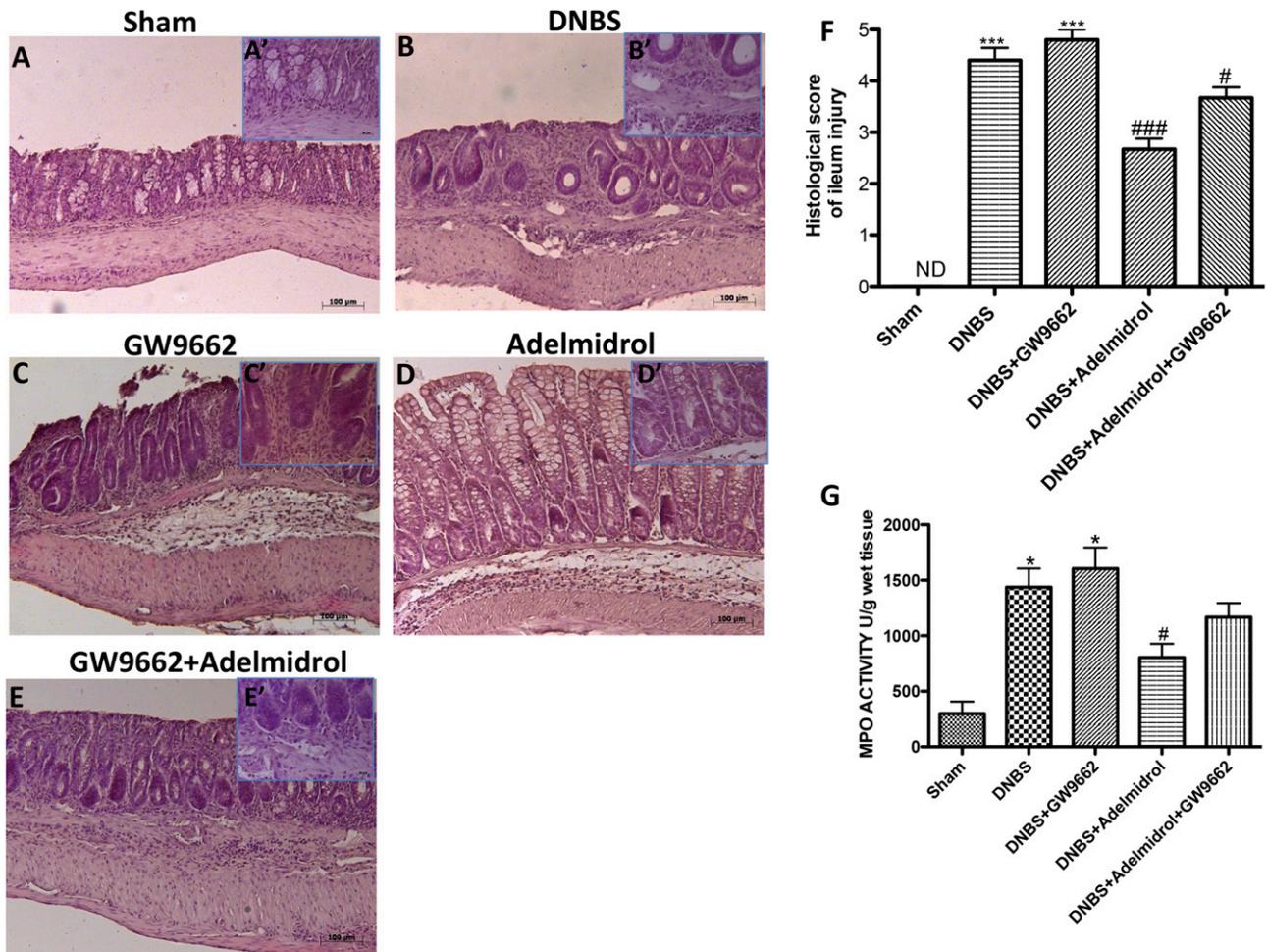


Fig. 9. Role of PPAR γ receptors on histologic damage and MPO activity compared with adelmidrol. Four days after intracolonic administration of DNBS, the colon presented a transmural necrosis and edema with a diffuse leukocyte cellular infiltrate in the submucosa (B,B'; histologic score (F) compared with sham (A, A'; histologic score F). No significant difference was appreciated between DNBS and DNBS + GW9662 group (C,C'); view histologic score (F). Adelmidrol (10 mg/kg, o.s.) reduced significantly the histologic signs of colon injury (D,D'); histologic score (F). The protective effects of adelmidrol (10 mg/kg, o.s.) were inhibited in mice injected with DNBS and pretreated with the PPAR γ antagonist GW9662, in terms of both histologic damage (E,E'); histologic score (F) and MPO activity (G). * $P < 0.05$ versus sham group; *** $P < 0.001$ versus sham group; # $P < 0.05$ versus DNBS group; ### $P < 0.001$ versus DNBS group.

TNF- α and IL-1 β are the most important cytokines present in colon tissues during the evolution of colitis (Kapsoritakis et al., 2001). The present study confirmed expression of TNF- α and IL-1 β in colon tissues from DNBS-injected mice. Importantly, treatment with adelmidrol reduced these inflammatory cytokine levels. It has been suggested that during acute and chronic colitis, the continuous production of proinflammatory cytokines plays a key role in the adherence and infiltration of leukocytes to endothelial cells, thus maintaining chronic inflammation within the cecal and colonic interstitium (Kawachi et al., 2000). Here, we confirmed that DNBS injection induced the manifestation of P-selectin on the endothelial vascular wall and upregulated surface expression of ICAM-1 on endothelial cells. Treatment with adelmidrol halted expression of P-selectin and ICAM-1 upregulation without effecting constitutive levels of ICAM-1 on endothelial cells. Activated neutrophils produce ROS and reactive nitrogen species within intestinal mucosa, which provoke oxidative stress, leading to DNA single-strand damage, PAR synthetase activation, and cell death (Szabó and Dawson, 1998; Mandalari et al., 2011). In accord with this process, we detected an increased staining for nitrotyrosine and

PAR in DNBS-injected mice, which was blunted by adelmidrol treatment. One form of epithelial cell damage in the inflamed colonic mucosa has been described to involve apoptosis (Araki et al., 2010). Interestingly, proapoptotic transcriptional changes, including upregulation of proapoptotic Bax and downregulation of antiapoptotic Bcl-2, were observed by Western blot. Treatment with adelmidrol lowered the signal for Bax compared with DNBS injected mice, whereas the signal for Bcl-2 in DNBS mice treated with adelmidrol was significantly greater than in the DNBS group.

As described herein, adelmidrol shares similar pharmacologic properties with PEA; this can be related to activation of nuclear receptor of PPAR family (Farquhar-Smith et al., 2002; Paterniti et al., 2013). Our previous studies, in fact, have proven that the deletion of PPAR genes reduced the protective effect of PEA; additionally, we found that PPAR α , PPAR γ , and PPAR δ agonists exerted positive effects in several experimental models, such as kidney ischemia/reperfusion and spinal cord trauma (Di Paola et al., 2012; Paterniti et al., 2013). Therefore, we have demonstrated that the anti-inflammatory effect of adelmidrol appeared to be related to PPAR γ

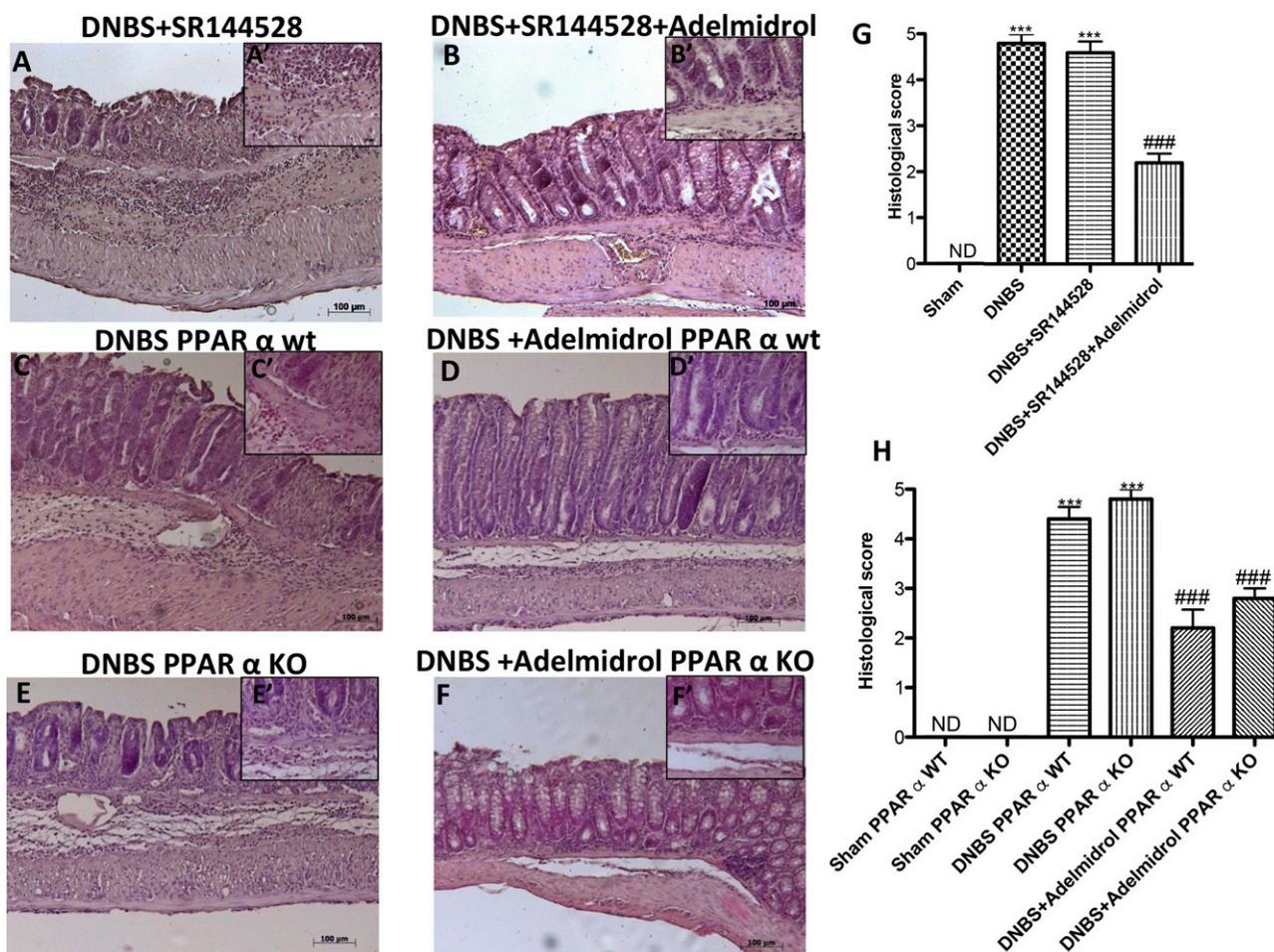


Fig. 10. Role of PPAR α and CB2 receptors in the systemic anti-inflammatory effects of adelmidrol in DNBS-induced colitis. As showed in panel 10, a transmural necrosis and edema with a diffuse leukocyte cellular infiltrate in the submucosa was found in the colon sections from DNBS-injected mice (A, A'). (C, C' and E, E') histologic score (G and H) compared with sham (data not shown). Adelmidrol (10 mg/kg, o.s.) reduced significantly the histologic signs of colon injury (B, B'; D, D' and F, F'); histologic score (G and H). * $P < 0.05$ versus sham group; *** $P < 0.001$ versus sham group; # $P < 0.05$ versus DNBS group; ### $P < 0.001$ versus DNBS group.

activation because the PPAR γ antagonist GW9662 efficiently neutralized the protective actions of adelmidrol against DNBS-induced colitis. On the contrary, use of PPAR α KO mice has clearly shown that the adelmidrol protective effect is not associated with PPAR α activation. Our results are also in agreement with a previous study in which the administration of GW6471, a PPAR α antagonist, did not reverse the anti-inflammatory effect of adelmidrol (De Filippis et al., 2009). Finally, we have also showed, using the CB2 antagonist SR144528, that the adelmidrol protective effect is not related to the activation of the CB2 receptor pathways.

Our study clearly has established that the use of adelmidrol, compared with PEA, presents some important differences that may clearly justify its use in chronic inflammatory disease. In particular, it has been demonstrated that adelmidrol, unlike than PEA, which is very lipophilic, presents both hydrophilic and lipophilic features; moreover, adelmidrol increases PEA intracellular concentrations, modulating the availability of endogenous anti-inflammatory lipids (Petrosino et al., 2016). In addition, our study clearly demonstrates in this experimental model that adelmidrol, in contrast with PEA, exerts its anti-inflammatory properties by actions only on the PPAR γ receptor, without involvement of the PPAR α or CB2 receptor

related pathways (Borrelli et al., 2015; Impellizzeri et al., 2015b). This evidence clearly represents a specific molecular mechanism that differentiates adelmidrol treatment from PEA. In conclusion, adelmidrol could represent a novel therapeutic intervention in autoimmune/inflammatory disorders such as IBD.

Acknowledgments

Salvatore Cuzzocrea is coinventor on patent WO2013121449 A8 (Epitech group SpA), which deals with compositions and methods for the modulation of amidases capable of hydrolysing N-acyl ethanolamines usable in the therapy of inflammatory diseases as well as a coinventor on a patent just submitted entitled "Adelmidrol per l'uso nelle patologie caratterizzate da insufficiente agonismo del recettore ppargamma," no. 102015000067344 del 30/10/2015 (Italy) (Epitech group SpA). Moreover, Dr. Cuzzocrea is also a coinventor with Epitech group on the following patents: EP 2 821 083, MI2014 A001495, 102015000067344

Authorship Contributions

Participated in research design: Esposito, Cuzzocrea.
 Conducted experiments: Impellizzeri, Gugliandolo, Siracusa.
 Performed data analysis: Crupi.
 Wrote or contributed to the writing of the manuscript: Cordaro.

References

- Aloe L, Leon A, and Levi-Montalcini R (1993) A proposed autacoid mechanism controlling mastocyte behaviour. *Agents Actions* **39**:C145–C147.
- Araki Y, Mukaiyso K, Sugihara H, Fujiyama Y, and Hattori T (2010) Increased apoptosis and decreased proliferation of colonic epithelium in dextran sulfate sodium-induced colitis in mice. *Oncol Rep* **24**:869–874.
- Baumgart DC and Sandborn WJ (2012) Crohn's disease. *Lancet* **380**:1590–1605.
- Bethea JR, Castro M, Keane RW, Lee TT, Dietrich WD, and Zezienski RP (1998) Traumatic spinal cord injury induces nuclear factor-kappaB activation. *J Neurosci* **18**:3251–3260.
- Borrelli F, Romano B, Petrosino S, Pagano E, Capasso R, Coppola D, Battista G, Orlando P, Di Marzo V, and Izzo AA (2015) Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal anti-inflammatory agent. *Br J Pharmacol* **172**:142–158.
- Bowie A and O'Neill LA (2000) Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* **59**:13–23.
- Casili G, Cordaro M, Impellizzeri D, Bruschetta G, Paterniti I, Cuzzocrea S, and Esposito E (2016) Dimethyl fumarate reduces inflammatory responses in experimental colitis. *J Crohn's Colitis* **10**:472–483.
- Costa B, Comelli F, Bettoni I, Colloeni M, and Giagnoni G (2008) The endogenous fatty acid amide, palmitoylethanolamide, has anti-allodynic and anti-hyperalgesic effects in a murine model of neuropathic pain: involvement of CB(1), TRPV1 and PPARgamma receptors and neurotrophic factors. *Pain* **139**:541–550.
- Cuzzocrea S, Di Paola R, Mazzon E, Genovese T, Muià C, Centorrino T, and Caputi AP (2004) Role of endogenous and exogenous ligands for the peroxisome proliferator-activated receptors alpha (PPAR-alpha) in the development of inflammatory bowel disease in mice. *Lab Invest* **84**:1643–1654.
- Cuzzocrea S, Ianaro A, Wayman NS, Mazzon E, Pisano B, Dugo L, Serraino I, Di Paola R, Chatterjee PK, Di Rosa M, et al. (2003) The cyclopentenone prostaglandin 15-deoxy-delta(12,14)-PGJ2 attenuates the development of colon injury caused by dinitrobenzene sulphonic acid in the rat. *Br J Pharmacol* **138**:678–688.
- D'Agostino G, La Rana G, Russo R, Sasso O, Iacono A, Esposito E, Raso GM, Cuzzocrea S, Lo Verme J, Piomelli D, et al. (2007) Acute intracerebroventricular administration of palmitoylethanolamide, an endogenous peroxisome proliferator-activated receptor-alpha agonist, modulates carrageenan-induced paw edema in mice. *J Pharmacol Exp Ther* **322**:1137–1143.
- De Filippis D, D'Amico A, Cinelli MP, Esposito G, Di Marzo V, and Iuvone T (2009) Adelmidrol, a palmitoylethanolamide analogue, reduces chronic inflammation in a carrageenin-granuloma model in rats. *J Cell Mol Med* **13**:1086–1095.
- De Filippis D, Luongo L, Cipriano M, Palazzo E, Cinelli MP, de Novellis V, Maione S, and Iuvone T (2011) Palmitoylethanolamide reduces granuloma-induced hyperalgesia by modulation of mast cell activation in rats. *Mol Pain* **7**:3–11.
- Di Paola R, Impellizzeri D, Mondello P, Velardi E, Aloisi C, Cappellani A, Esposito E, and Cuzzocrea S (2012) Palmitoylethanolamide reduces early renal dysfunction and injury caused by experimental ischemia and reperfusion in mice. *Shock* **38**:356–366.
- Esposito E, Paterniti I, Mazzon E, Genovese T, Di Paola R, Galuppo M, and Cuzzocrea S (2011) Effects of palmitoylethanolamide on release of mast cell peptidases and neurotrophic factors after spinal cord injury. *Brain Behav Immun* **25**:1099–1112.
- Farquhar-Smith WP, Jaggard SI, and Rice AS (2002) Attenuation of nerve growth factor-induced visceral hyperalgesia via cannabinoid CB(1) and CB(2)-like receptors. *Pain* **97**:11–21.
- Franklin A, Parmentier-Batteur S, Walter L, Greenberg DA, and Stella N (2003) Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. *J Neurosci* **23**:7767–7775.
- Genovese T, Esposito E, Mazzon E, Di Paola R, Meli R, Bramanti P, Piomelli D, Calignano A, and Cuzzocrea S (2008) Effects of palmitoylethanolamide on signaling pathways implicated in the development of spinal cord injury. *J Pharmacol Exp Ther* **326**:12–23.
- Halliwell B (1997) Antioxidants and human disease: a general introduction. *Nutrition Rev* **55**(1 Pt 2): S44–S52.
- Hansen HS, Moesgaard B, Petersen G, and Hansen HH (2002) Putative neuroprotective actions of N-acyl-ethanolamines. *Pharmacol Ther* **95**:119–126.
- Impellizzeri D, Bruschetta G, Di Paola R, Ahmad A, Campolo M, Cuzzocrea S, Esposito E, and Navarra M (2015a) The anti-inflammatory and antioxidant effects of bergamot juice extract (Bje) in an experimental model of inflammatory bowel disease. *Clin Nutr* **34**:1146–1154.
- Impellizzeri D, Campolo M, Di Paola R, Bruschetta G, de Stefano D, Esposito E, and Cuzzocrea S (2015b) Ultramicrodosed palmitoylethanolamide reduces inflammation in a Th1-mediated model of colitis. *Eur J Inflamm* **13**:14–31.
- Kapsoritakis AN, Koukourakis MI, Sfridakis A, Potamianos SP, Kosmadaki MG, Koutroubakis IE, and Kouroumalis EA (2001) Mean platelet volume: a useful marker of inflammatory bowel disease activity. *Am J Gastroenterol* **96**:776–781.
- Kawachi S, Jennings S, Panes J, Cockrell A, Laroux FS, Gray L, Perry M, van der Heyde H, Balish E, Granger DN, et al. (2000) Cytokine and endothelial cell adhesion molecule expression in interleukin-10-deficient mice. *Am J Physiol Gastrointest Liver Physiol* **278**:G734–G743.
- Koizumi M, King N, Lobb R, Benjamin C, and Podolsky DK (1992) Expression of vascular adhesion molecules in inflammatory bowel disease. *Gastroenterology* **103**: 840–847.
- Longo WE, Panesar N, Mazuski J, and Kaminski DL (1998) Contribution of cyclooxygenase-1 and cyclooxygenase-2 to prostanoid formation by human enterocytes stimulated by calcium ionophore and inflammatory agents. *Prostaglandins Other Lipid Mediat* **56**:325–339.
- Mandalari G, Bisignano C, Genovese T, Mazzon E, Wickham MS, Paterniti I, and Cuzzocrea S (2011) Natural almond skin reduced oxidative stress and inflammation in an experimental model of inflammatory bowel disease. *Int Immunopharmacol* **11**:915–924.
- Mastrofrancesco A, Ottaviani M, Aspate N, Cardinali G, Izzo E, Graupe K, Zouboulis CC, Camera E, and Picardo M (2010) Azelaic acid modulates the inflammatory response in normal human keratinocytes through PPARgamma activation. *Exp Dermatol* **19**:813–820.
- Mullane KM, Kraemer R, and Smith B (1985) Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J Pharmacol Methods* **14**:157–167.
- Nagayama T, Sinor AD, Simon RP, Chen J, Graham SH, Jin K, and Greenberg DA (1999) Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J Neurosci* **19**:2987–2995.
- Ordás I, Eckmann L, Talamini M, Baumgart DC, and Sandborn WJ (2012) Ulcerative colitis. *Lancet* **380**:1606–1619.
- Paterniti I, Impellizzeri D, Crupi R, Morabito R, Campolo M, Esposito E, and Cuzzocrea S (2013) Molecular evidence for the involvement of PPAR-δ and PPAR-γ in anti-inflammatory and neuroprotective activities of palmitoylethanolamide after spinal cord trauma. *J Neuroinflammation* **10**:20–32.
- Petrosino S, Puigdemont A, Della Valle MF, Fusco M, Verde R, Allarà M, Aveta T, Orlando P, and Di Marzo V (2016) Adelmidrol increases the endogenous concentrations of palmitoylethanolamide in canine keratinocytes and down-regulates an inflammatory reaction in an in vitro model of contact allergic dermatitis. *Vet J* **207**: 85–91.
- Pierik M, Yang H, Barmada MM, Cavanaugh JA, Annesse V, Brant SR, Cho JH, Duerr RH, Hugot JP, McGovern DP, et al.; IBD International Genetics Consortium (2005) The IBD international genetics consortium provides further evidence for linkage to IBD4 and shows gene-environment interaction. *Inflamm Bowel Dis* **11**: 1–7.
- Pulvirenti N, Nasca MR, and Micali G (2007) Topical adelmidrol 2% emulsion, a novel aliamide, in the treatment of mild atopic dermatitis in pediatric subjects: a pilot study. *Acta Dermatovenerol Croat* **15**:80–83.
- Reddy SN, Bazzocchi G, Chan S, Akashi K, Villanueva-Meyer J, Yanni G, Mena I, and Snape WJ, Jr (1991) Colonic motility and transit in health and ulcerative colitis. *Gastroenterology* **101**:1289–1297.
- Riordan AM, Ruxton CH, and Hunter JO (1998) A review of associations between Crohn's disease and consumption of sugars. *Eur J Clin Nutr* **52**:229–238.
- Sandborn WJ and Hanauer SB (1999) Antitumor necrosis factor therapy for inflammatory bowel disease: a review of agents, pharmacology, clinical results, and safety. *Inflamm Bowel Dis* **5**:119–133.
- Schmid HH and Berdyshev EV (2002) Cannabinoid receptor-inactive N-acyl-ethanolamines and other fatty acid amides: metabolism and function. *Prostaglandins Leukot Essent Fatty Acids* **66**:363–376.
- Scuderi C, Valenza M, Stecca C, Esposito G, Carratù MR, and Steardo L (2012) Palmitoylethanolamide exerts neuroprotective effects in mixed neuroglial cultures and organotypic hippocampal slices via peroxisome proliferator-activated receptor-α. *J Neuroinflammation* **9**:43–49.
- Shea TB (1994) Technical report. An inexpensive densitometric analysis system using a Macintosh computer and a desktop scanner. *Biotechniques* **16**:1126–1128.
- Skaper SD, Facci L, Romanello S, and Leon A (1996) Mast cell activation causes delayed neurodegeneration in mixed hippocampal cultures via the nitric oxide pathway. *J Neurochem* **66**:1157–1166.
- Szabó C and Dawson VL (1998) Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. *Trends Pharmacol Sci* **19**:287–298.
- Wendland BE, Aghdassi E, Tam C, Carrier J, Steinhart AH, Wolman SL, Baron D, and Allard JP (2001) Lipid peroxidation and plasma antioxidant micronutrients in Crohn disease. *Am J Clin Nutr* **74**:259–264.
- Ye Y, Pang Z, Chen W, Ju S, and Zhou C (2015) The epidemiology and risk factors of inflammatory bowel disease. *Int J Clin Exp Med* **8**:22529–22542.
- Zingarelli B, Squadrito F, Graziani P, Camerini R, and Caputi AP (1993) Effects of zileuton, a new 5-lipoxygenase inhibitor, in experimentally induced colitis in rats. *Agents Actions* **39**:150–156.

Address correspondence to: Prof. Salvatore Cuzzocrea, Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale Ferdinando Stagno D'Alcontres n°31 98166 Messina, Italy. E-mail: salvator@unime.it