

RESEARCH PAPER

Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal anti-inflammatory agent

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BACKGROUND AND PURPOSE

Palmitoylethanolamide (PEA) acts via several targets, including cannabinoid CB₁ and CB₂ receptors, transient receptor potential vanilloid type-1 (TRPV1) ion channels, peroxisome proliferator-activated receptor alpha (PPAR α) and orphan G protein-coupled receptor 55 (GPR55), all involved in the control of intestinal inflammation. Here, we investigated the effect of PEA in a murine model of colitis.

EXPERIMENTAL APPROACH

Colitis was induced in mice by intracolonic administration of dinitrobenzenesulfonic acid (DNBS). Inflammation was assessed by evaluating inflammatory markers/parameters and by histology; intestinal permeability by a fluorescent method; colonic cell proliferation by immunohistochemistry; PEA and endocannabinoid levels by liquid chromatography mass spectrometry; receptor and enzyme mRNA expression by quantitative RT-PCR.

KEY RESULTS

DNBS administration caused inflammatory damage, increased colonic levels of PEA and endocannabinoids, down-regulation of mRNA for TRPV1 and GPR55 but no changes in mRNA for CB₁, CB₂ and PPAR α . Exogenous PEA (i.p. and/or p.o., 1 mg·kg⁻¹) attenuated inflammation and intestinal permeability, stimulated colonic cell proliferation, and increased colonic TRPV1 and CB₁ receptor expression. The anti-inflammatory effect of PEA was attenuated or abolished by CB₂ receptor, GPR55 or PPAR α antagonists and further increased by the TRPV1 antagonist capsazepine.

CONCLUSIONS AND IMPLICATIONS

PEA improves murine experimental colitis, the effect being mediated by CB₂ receptors, GPR55 and PPAR α , and modulated by TRPV1 channels.

Abbreviations

2-AG, 2-arachidonoylglycerol; CB, cannabinoid; DNBS, 2,4,6-dinitrobenzenesulfonic acid; DSS, dextran sodium sulphate; FAAH, fatty acid amide hydrolase; GDE1, glycerophosphodiester PDE 1; IBD, inflammatory bowel disease; MPO, myeloperoxidase; NAAA, N-acylethanolamine-hydrolysing acid amidase; NAPE-PLD, N-arachidonyl-phosphatidylethanolamine PLD; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; TRPV1, transient receptor potential vanilloid type-1

Tables of Links

TARGETS
GPCRs^a
CB ₁ receptor
CB ₂ receptor
GPR55
Ion Channels^b
TRPV1
Nuclear receptors^c
PPAR α
Enzymes^d
FAAH, fatty acid amide hydrolase
NAAA, <i>N</i> -acylethanolamine acid amidase
NAPE-PLD, <i>N</i> -arachidonyl-phosphatidylethanolamine PLD

LIGANDS
2-AG, 2-arachidonoylglycerol
AM630
Anandamide
Capsazepine
GW6471
OEA, oleoylethanolamide
PEA, palmitoylethanolamide
Rimonabant

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b,c,d}Alexander *et al.*, 2013a,b,c,d).

Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, is a chronic relapsing and destructive inflammatory disorder of the gastrointestinal tract. Significant progress in understanding IBD pathophysiology has led to the development of new therapies that target key molecules and immunological mechanisms (Löwenberg and D'Haens, 2013). However, the corticosteroids and the immunomodulatory drugs, which are the basis of treatment for the IBDs, do not always provide satisfactory outcomes (Burger and Travis, 2011; Ioannidis *et al.*, 2011). Probably for this reason, the use of nutraceutical supplements by patients with IBD is widespread and growing. Recent studies agree that approximately 50% of patients with IBD try non-conventional remedies, which include the use of nutraceuticals (Opheim *et al.*, 2012; Koning *et al.*, 2013).

Palmitoylethanolamide (PEA) is a food component first discovered in the late 1950s when it was shown that the anti-allergic and anti-inflammatory activity exerted by egg yolk, peanut oil or soybean lecithin was due to a specific lipid fraction corresponding to PEA (Esposito and Cuzzocrea, 2013). In Italy and Spain, PEA is actually marketed under the brand name Normast (Epitech Srl, Milan, Italy) as a food component for special medical purposes to alleviate bowel complaints, although randomized clinical trials are lacking. Additionally, in the United States, PEA preparations are promoted for the treatment of IBD (proposed brand name Recoclix, CM&D Pharma Ltd., Nestlé) (Keppel Hesselink *et al.*, 2013).

Apart from being present in the plant kingdom, PEA is biosynthesized and metabolized by different animal cell types (Keppel Hesselink *et al.*, 2013). Chemically, PEA belongs to the family of acylethanolamides that include the endocannabinoid anandamide and the anorectic mediator oleoylethanolamide (OEA). Anandamide, OEA and PEA share anabolic and catabolic pathways with glycerophosphodiester

PDE 1 (GDE1) and *N*-arachidonyl-phosphatidylethanolamine PLD (NAPE-PLD) being involved in their biosynthesis and fatty acid amide hydrolase (FAAH) in their degradation (Petrosino *et al.*, 2010; Blankman and Cravatt, 2013). Additionally, *N*-acylethanolamine-hydrolysing acid amidase (NAAA) has been recently identified as a key specific enzyme involved in PEA degradation (Tsuboi *et al.*, 2007). It is now established that PEA is biosynthesized to maintain cellular homeostasis when this is challenged by external stressors provoking inflammation, neuronal damage and pain (Skaper and Facci, 2012). PEA has been identified in the rodent (Capasso *et al.*, 2001; Fu *et al.*, 2007; Izzo *et al.*, 2010; Diep *et al.*, 2011) and human (Darmani *et al.*, 2005; D'Argenio *et al.*, 2007) digestive tract and, given exogenously (i.p.), inhibits intestinal transit in the inflamed gut (Capasso *et al.*, 2001) and reduces intestinal injury caused by ischaemia-reperfusion (Di Paola *et al.*, 2012). Proposed direct or indirect targets for PEA actions include a number of receptors, namely, cannabinoid (CB)₁ and CB₂ receptors (De Petrocellis *et al.*, 2002; Smart *et al.*, 2002), transient receptor potential vanilloid type-1 (TRPV1) ion channels (Ambrosino *et al.*, 2013), PPAR α (Lo Verme *et al.*, 2005) and the orphan receptor GPR55 (Pertwee, 2007), which are involved (in the case of CB₁, CB₂, TRPV1 and PPAR α) or are advocated to be involved (i.e. GPR55) in the mechanisms controlling intestinal inflammation (Cuzzocrea *et al.*, 2004; Izzo and Camilleri, 2009; Holzer, 2011; Schicho *et al.*, 2011).

Because PEA acts on key targets regulating intestinal inflammation and in the light of the widespread use of PEA-containing over-the-counter preparations for the relief of intestinal complaints, including IBD, we have investigated here the effect and the mode of action of this acylethanolamide in a murine model of colitis. Preliminary accounts of some of these results have been communicated to the XXXVI National Congress of the Italian Pharmacological Society (Petrosino *et al.*, 2013).

Methods

Animals

All animal care and experimental procedures complied with the principles of laboratory animal care (NIH publication no.86-23, revised 1985) and the Italian D.L. no.116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 402 animals were used in the experiments described here.

Male ICR mice, weighing 25–30 g, were purchased by Harlan Italy (Corezzana, Milan, Italy) and housed in polycarbonate cages in isolators under a 12 h light/12 h dark cycle, temperature $23 \pm 2^\circ\text{C}$ and humidity 60%. Mice were fed *ad libitum* with standard food, except for the 24 h period immediately preceding the administration of 2,4,6-dinitrobenzenesulfonic acid (DNBS) and for the 2 h period preceding the administration of p.o. PEA.

Induction of experimental colitis and pharmacological treatment

Colitis was induced by the intracolonic administration of DNBS (Romano *et al.*, 2013). Briefly, mice were anaesthetized with inhaled 5% isoflurane (Centro Agrovete Campania, Scafati, SA, Italy) and subsequently DNBS ($150 \text{ mg}\cdot\text{kg}^{-1}$) was injected into the colon using a polyethylene catheter (1 mm in diameter) via the rectum (4.5 cm from the anus). Control mice received an intracolonic administration of water. Three days after DNBS or water administration, all animals were killed by asphyxiation with CO_2 , the mice abdomen was opened by a midline incision and the colon removed, isolated from surrounding tissues, opened along the anti-mesenteric border, rinsed, weighed and the length measured in order to determine the colon weight/colon length ratio ($\text{mg}\cdot\text{cm}^{-1}$) used as an indirect marker of inflammation. The body weight of mice was measured every day throughout the treatment period. All measurements were performed by workers who were unaware of the particular treatment. For biochemistry analysis, tissues were kept at -80°C until use, whereas for histological examination tissues were fixed in 10% formaldehyde. The dose of DNBS was selected on the basis of preliminary experiments showing colonic damage associated with high reproducibility and low mortality for a $150 \text{ mg}\cdot\text{kg}^{-1}$ dose. The time point of damage evaluation (i.e. 3 days after DNBS administration) was chosen because maximal DNBS-induced inflammation has been reported in mice after 3 days (Massa *et al.*, 2004). PEA ($0.1\text{--}10 \text{ mg}\cdot\text{kg}^{-1}$) was administered i.p. or p.o. three times (once a day) for three consecutive days starting 24 h after DNBS or vehicle inoculation (therapeutic protocol). Animals were killed 2 h after the third administration of PEA. In some experiments, the effect of PEA ($1 \text{ mg}\cdot\text{kg}^{-1}$, p.o.) was evaluated in animals pretreated every day (i.p., 30 min before PEA) with the CB_1 receptor antagonist rimonabant ($3 \text{ mg}\cdot\text{kg}^{-1}$), the CB_2 antagonist AM630 ($10 \text{ mg}\cdot\text{kg}^{-1}$), the PPAR α antagonist GW6471 ($1 \text{ mg}\cdot\text{kg}^{-1}$), the GPR55 antagonist ML-191 ($0.5 \text{ mg}\cdot\text{kg}^{-1}$) or the TRPV1 channels antagonist capsazepine ($10 \text{ mg}\cdot\text{kg}^{-1}$). The

doses of rimonabant, AM630, GW6471 and capsazepine were selected on the basis of previous published work concerning gastrointestinal pharmacology (De Schepper *et al.*, 2008; Alhouayek *et al.*, 2011; Capasso *et al.*, 2014). The dose of ML-191 was selected on the basis of preliminary experiments showing that the antagonist, at $0.5 \text{ mg}\cdot\text{kg}^{-1}$ dose, did not affect, *per se*, DNBS-induced intestinal inflammation. A higher dose of ML-191 ($1 \text{ mg}\cdot\text{kg}^{-1}$) affected, *per se*, DNBS-induced colitis (see Results).

Histology and immunohistochemistry

All histological and immunochemical evaluation was carried out without knowledge of the treatments. Such evaluations, performed 3 days after DNBS administration, were assessed on a segment of 1 cm of colon taken from 4 cm above the anal canal. After fixation for 24 h in saline 10% formaldehyde, samples were dehydrated in graded ethanol and embedded in paraffin. Thereafter, 5 mm sections were deparaffinized with xylene, stained with haematoxylin–eosin and observed in a DM 4000 B Leica microscope (Leica Microsystems, Milan, Italy). Microscopic scoring was performed as previously reported (Romano *et al.*, 2013). Briefly, the colon was scored considering (i) submucosal infiltration (0, none; 1, mild; 2–3, moderate; 4–5 severe); (ii) crypt abscesses (0, none; 1–2 rare; 3–5, diffuse); and (iii) mucosal erosion (0, absent; 1, focus; 2–3, extended until the middle of the visible surface; 4–5, extended until the entire visible surface).

For immunohistochemical detection of Ki-67, paraffin-embedded slides were immersed in a Tris/EDTA buffer (pH 9.0), heated in a decloaking chamber at 125°C for 3 min and cooled at room temperature for 20 min. Sections were incubated for 10 min with 3% hydrogen peroxide, washed with Tris-buffered saline Tween 20 (Sigma-Aldrich, Milan, Italy) (pH 7.6) and incubated with rabbit monoclonal antibody to Ki-67 (1:100, Ventana Medical Systems, Tucson, AZ, USA) for 30 min at room temperature. The slides were washed three times with Tris-buffered saline Tween 20 and incubated with secondary antibody for 30 min. After, the slides were reacted with streptavidin for 20 min and 3,3'-diaminobenzidine tetrahydrochloride for 5 min. Finally, the slides were counterstained with Mayer's haematoxylin. The intensity and localization of immunoreactivities against the primary antibody were examined on all sections with a microscope (Leica Microsystems). To ensure specificity of the immunohistochemical procedure, some tissues were processed without primary antibody to Ki-67.

Intestinal permeability assay

Intestinal permeability was evaluated using a FITC-labelled dextran method, as described previously (Borrelli *et al.*, 2013). Briefly, 2 days after DNBS administration, mice were gavaged with $600 \text{ mg}\cdot\text{kg}^{-1}$ body weight of FITC-conjugated dextran (molecular mass 3–5 kDa). One day later, blood was collected by cardiac puncture, and the serum was immediately analysed for FITC-derived fluorescence (fluorescent microplate reader with 2104 EnVision Multilabel Plate Readers, [PerkinElmer Instruments, Waltham, MA, USA]; excitation wavelengths $485 \pm 14 \text{ nm}$, emission wavelengths $520 \pm 25 \text{ nm}$). Serial-diluted FITC dextran was used to generate a standard curve. Intestinal permeability was expressed as the concentrations of FITC (μM) found in the serum.

Myeloperoxidase (MPO) activity

MPO activity, a marker used to quantify the extent of neutrophil accumulation in whole-tissue colons, was determined as previously described (Borrelli *et al.*, 2013). Full-thickness colons were homogenized in an appropriate lysis buffer (0.5% hexadecyltrimethylammonium bromide in 3-(N-morpholino)propanesulfonic acid [MOPS] 10 mM) in the ratio of 50 mg tissue per mL MOPS. The samples were then centrifuged for 20 min at $15\,000\times g$ at 4°C. An aliquot of the supernatant was then incubated with NaPP (sodium phosphate buffer pH 5.5) and tetra-methylbenzidine 16 mM. After 5 min, H₂O₂ (9.8 M) in NaPP was added and the reaction stopped with acetic acid. The rate of change in absorbance was measured by a spectrophotometer at 650 nm. Different dilutions of human MPO enzyme of known concentration were used to obtain a standard curve. MPO activity was expressed as U·mg⁻¹ of tissue.

Quantitative (real-time) RT-PCR analysis

The colons from animals treated with vehicles (control group), PEA, DNBS or DNBS plus PEA were removed (3 days after the administration of DNBS or water), collected in RNA later (Invitrogen, Carlsbad, CA, USA) and homogenized by a rotorstator homogenizer in 1.5 mL of Trizol® (Invitrogen). Total RNA was purified, quantified, characterized and retro-transcribed as previously described (Grimaldi *et al.*, 2009). For all samples tested, the RNA integrity number (Bionalyzer 2100, Agilent) was greater than eight relative to a 0–10 scale. Quantitative real-time PCR was performed by an iCycler-iQ5® (Bio-Rad, Milan, Italy) in a 20 µL reaction mixture as described. Assays were performed in quadruplicate (maximum ΔCt of replicate samples <0.5), and a standard curve from consecutive fivefold dilutions (100–0.16 ng) of a cDNA pool representative of all samples was included for PCR efficiency determination. Optimized primers for SYBR Green analysis and optimum annealing temperatures were designed by the Allele-Id software version 7.0 (Biosoft International, Palo Alto, CA, USA) and were synthesized (HPLC purification grade) by MWG-Biotech (Ebersberg, Germany). For each target, all mRNA sequences at <http://www.ncbi.nlm.nih.gov/gene/> were aligned and common primers were designed (see Supporting Information Table S1 for primer sequences). Relative expression calculation, correct for PCR efficiency and normalized with respect to reference genes β-actin and hypoxanthine-guanine phosphoribosyltransferase, was performed by the iQ5 software. Results are expressed as fold expression, compared with control (=1) (Aviello *et al.*, 2012). Statistical significance was evaluated by the REST 2009 software (Pfaffl *et al.*, 2002).

Identification and quantification of PEA, endocannabinoids and OEA

Full-thickness colons from animals receiving DNBS or vehicle (treated or not with PEA 1 mg·kg⁻¹, p.o.) were removed (3 days after DNBS administration), immediately immersed into liquid nitrogen and stored at -80°C until extraction of endocannabinoids [anandamide and 2-arachidonoylglycerol (2-AG)] and related acylethanolamides (PEA and OEA). Tissues were extracted with chloroform/methanol (2:1, by volume) containing each 10 pmol of d₈-anandamide, d₄-PEA,

d₄-OEA and d₅-2-AG, synthesized as described previously (for the former two compounds) (Di Marzo *et al.*, 2008), or provided by Cayman Chemicals (for d₅-2-AG, Ann Arbor, MI, USA). The lipid extracts were purified by silica column chromatography and the fractions containing anandamide, PEA and 2-AG were analysed by isotope dilution liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC-MS). Results were expressed as picomoles per milligram of tissue.

Data analysis

Data are expressed as the mean ± SEM of *n* experiments. To determine statistical significance, Student's *t*-test was used for comparing a single treatment mean with a control mean, and a one-way ANOVA followed by a Tukey–Kramer multiple comparisons test was used for the analysis of multiple treatment means. Values of *P* less than 0.05 were considered significant.

Materials

Ultramicronized PEA was kindly provided by Epitech Group (Saccolongo, Italy) and rimonabant by SANOFI Recherche, Montpellier, France. DNBS hydrate, MPO from human leucocytes and FITC-conjugated dextran (molecular mass 3–5 kDa) were purchased from Sigma Aldrich S.r.l. (Milan, Italy). Capsazepine, GW6471 and AM630 were supplied by Tocris (Space Import-Export SrL, Milan, Italy) and ML-191 by Cayman (Cabru SAS, Arcore, Italy). All reagents for cell culture and Western blot analysis were obtained from Sigma Aldrich S.r.l., Amersham Biosciences Inc. (Piscataway, NJ, USA), Bio-Rad Laboratories (Milan, Italy) and Microtech S.r.l. (Naples, Italy). All chemicals and reagents employed in this study were of analytical grade.

PEA was dissolved in ethanol/Tween 20/saline (1:1:8, 60 µL per mouse) for i.p. injection or suspended in carboxymethyl cellulose (1.5%, 150 µL per mouse) for p.o. administration. Rimonabant, ML-191 and capsazepine were dissolved in ethanol/Tween 20/saline (1:1:8, 60 µL per mouse), AM630 in DMSO/Tween 20/saline (1:1:8, 60 µL per mouse). DNBS was dissolved in 50% ethanol (0.15 mL per mouse). All vehicles had no significant effects on the responses under study.

Results

PEA reduced the impairment in body weight gain induced by colitis

Compared with control animals, DNBS administration caused significant weight loss. PEA (0.1–10 mg·kg⁻¹; i.p., Figure 1A or p.o., Figure 1B), administered after the inflammatory insult, reduced in a dose-dependent manner the loss of body weight induced by colitis, the effect being significant starting from the 0.3 mg·kg⁻¹ (i.p.) or 1 mg·kg⁻¹ (p.o.) doses (Figure 1A and B).

PEA-reduced colon weight/colon length ratio

Intracolonic DNBS administration caused inflammatory damage, as indicated by the approximately 2.5-fold increase in colon weight/colon length ratio (a simple and reliable marker of intestinal inflammation and/or damage) (Figure 1C

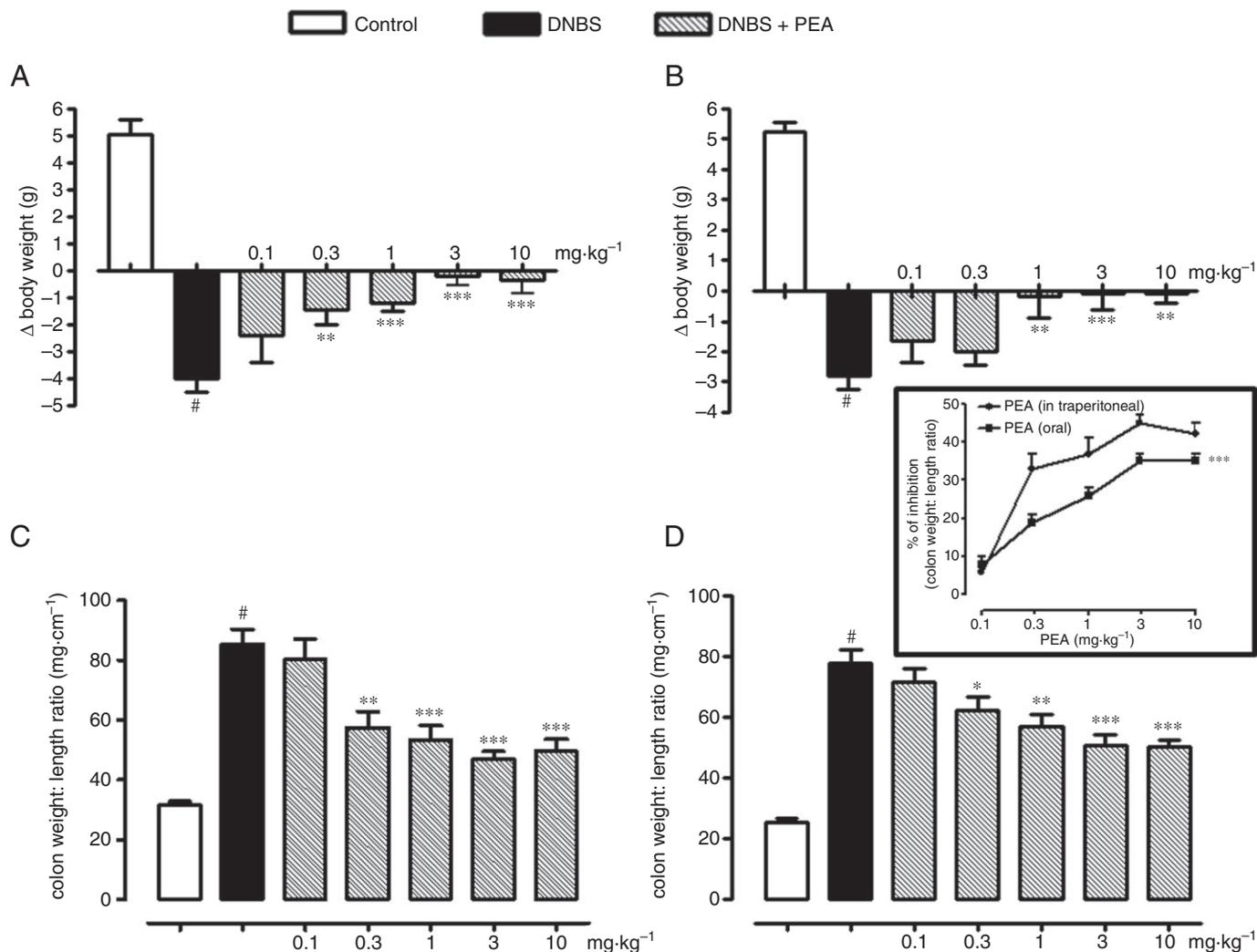


Figure 1

DNBS-induced colitis in mice. Changes in body weight (A,B) and colon weight/colon length ratio (C,D) from control and DNBS-treated mice in the presence or absence of i.p. (A,C) or p.o. (B,D) PEA. Mice were weighed before DNBS (or vehicle) administration and immediately before killing. Tissues were analysed 3 days after vehicle or DNBS administration. PEA (0.1–10 mg·kg⁻¹) was administered once a day for three consecutive days starting 24 h after the inflammatory insult (therapeutic protocol). Bars are mean ± SEM of 12–15 mice for each experimental group. #*P* < 0.001 versus control (i.e. mice without intestinal inflammation). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 versus DNBS alone. The insert reports the percentage of inhibition of colon weight/colon length ratio after i.p. or p.o. PEA administration and demonstrates that i.p. PEA was significantly (*P* < 0.001) more active than p.o. PEA in the experimental model of colitis induced by DNBS.

and D). PEA (0.1–10 mg·kg⁻¹; i.p., Figure 1C or p.o., Figure 1D), administered after the inflammatory insult, significantly and in a dose-dependent manner, reduced the effect of DNBS on colon weight/colon length ratio. Although a significant inhibition of this ratio was achieved starting from the PEA 0.3 mg·kg⁻¹ dose, irrespective of the route of administration, analysis of the curves representing the percentage of inhibition of inflammation showed a greater anti-inflammatory effect of PEA when given i.p. (see inset to Figure 1).

To confirm the anti-inflammatory activity of PEA, we performed MPO activity measurement, histological analysis, intestinal permeability measurement and immunohistochemistry using the submaximal dose of PEA of 1 mg·kg⁻¹.

PEA reduced microscopic damage

Histological analysis of colonic mucosa of control mice showed an intact, unbroken epithelium (Figure 2A and B). In the DNBS group, colons showed tissue injury, as revealed by necrosis involving the full thickness of the mucosa, infiltrations of granulocytes into the mucosa/submucosa, oedema of submucosa and intramuscular and subserosal inflammation (Figure 2C and D). PEA (1 mg·kg⁻¹, i.p. and p.o.) reduced the signs of colon injury. In the colon of animals given PEA (1 mg·kg⁻¹) (Figure 2E and F), the oedema and the erosion areas were reduced in the mucosa and in the submucosa [microscopic score: control, 0 ± 0; DNBS, 9.2 ± 0.6#; DNBS + PEA (i.p.) 3.3 ± 1.0**; DNBS + PEA (p.o.) 5.2 ± 0.5**; *n* = 4 for each experimental group; #*P* < 0.01 vs. control, ***P* < 0.01 vs. DNBS].

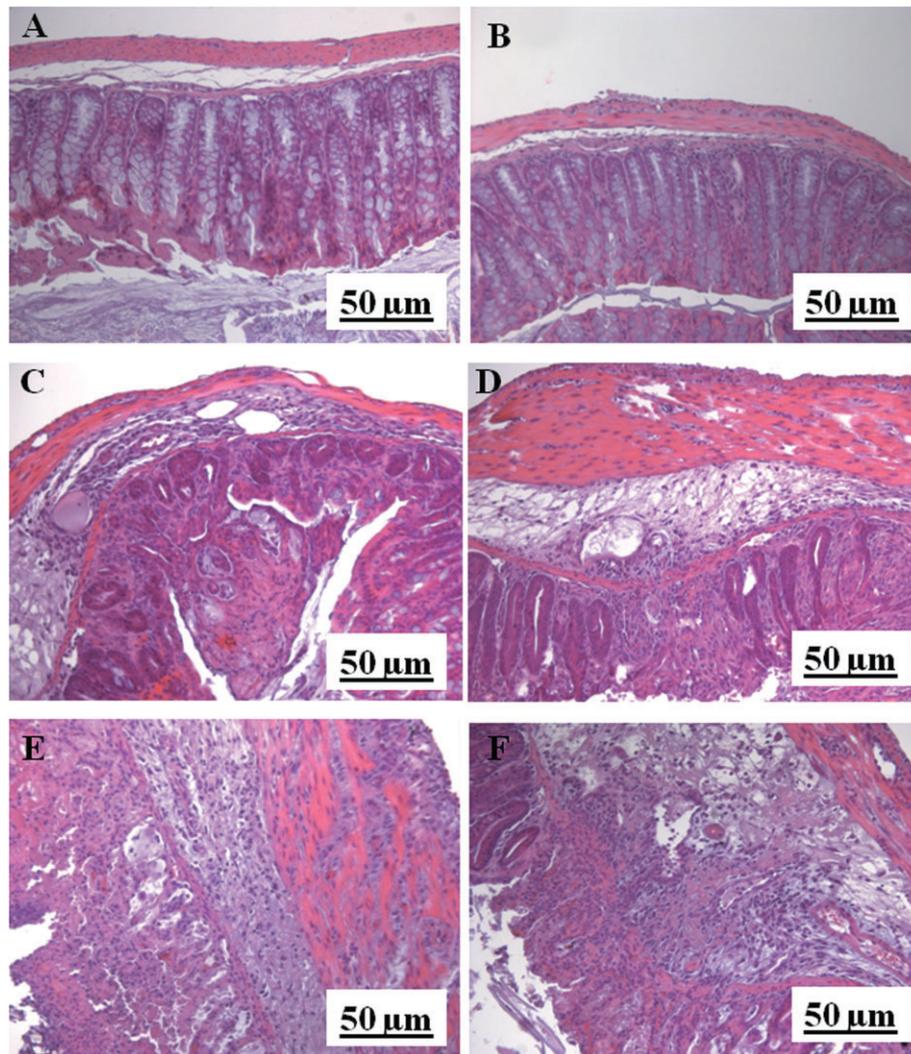


Figure 2

Histological evaluations of inflamed (DNBS treated) and non-inflamed colons: effect of PEA. No histological modification was observed in the mucosa and submucosa of control mice receiving the vehicle used to dissolve PEA i.p. (A) or p.o. (B); mucosal injury induced by DNBS administration in mice receiving the vehicle used to dissolve PEA i.p. (C) or p.o. (D); i.p. (E) and p.o. (F) PEA reduced the erosion and the inflammation area in the mucosa and in the submucosa. Histological analysis was performed 3 days after DNBS administration. PEA ($1 \text{ mg}\cdot\text{kg}^{-1}$) was administered once a day for three consecutive days starting 24 h after the inflammatory insult (therapeutic protocol). Original magnification $\times 200$. The Figure shows results representative of four experiments.

PEA reduced MPO activity

MPO activity is considered to be an index of neutrophil infiltration and it is largely used to quantify intestinal inflammation (Krawisz *et al.*, 1984). DNBS-induced colitis was associated with a significant increase in MPO activity, which was reduced by i.p. (Figure 3A, 60% inhibition) or p.o. (Figure 3B, 64% inhibition) administration of PEA ($1 \text{ mg}\cdot\text{kg}^{-1}$).

PEA reduced intestinal permeability

Intracolonic administration of DNBS increased intestinal permeability, as revealed by the abundant concentration of FITC-conjugated dextran in the serum. i.p. (Figure 3C) or p.o. (Figure 3D) PEA ($1 \text{ mg}\cdot\text{kg}^{-1}$) partially counteracted DNBS-

induced increase in intestinal permeability (57 and 47% inhibition, for i.p. and p.o. PEA administration respectively).

PEA stimulated colonic cell regeneration, as shown by immunohistochemistry

The action of PEA was further confirmed by immunohistochemistry. In normal colonic mucosa, the predominant area of cell proliferation was localized to the lower part of the crypts as revealed by Ki-67 distribution (Figure 4A and B). In the colon from DNBS-treated mice, total necrosis with Ki-67 immunoreactivity on inflammatory cells and in a few remaining surface elements was observed (Figure 4C and D). PEA ($1 \text{ mg}\cdot\text{kg}^{-1}$, i.p. and p.o.) partially counteracted the effect of DNBS on cell proliferation, its mitotic activity being

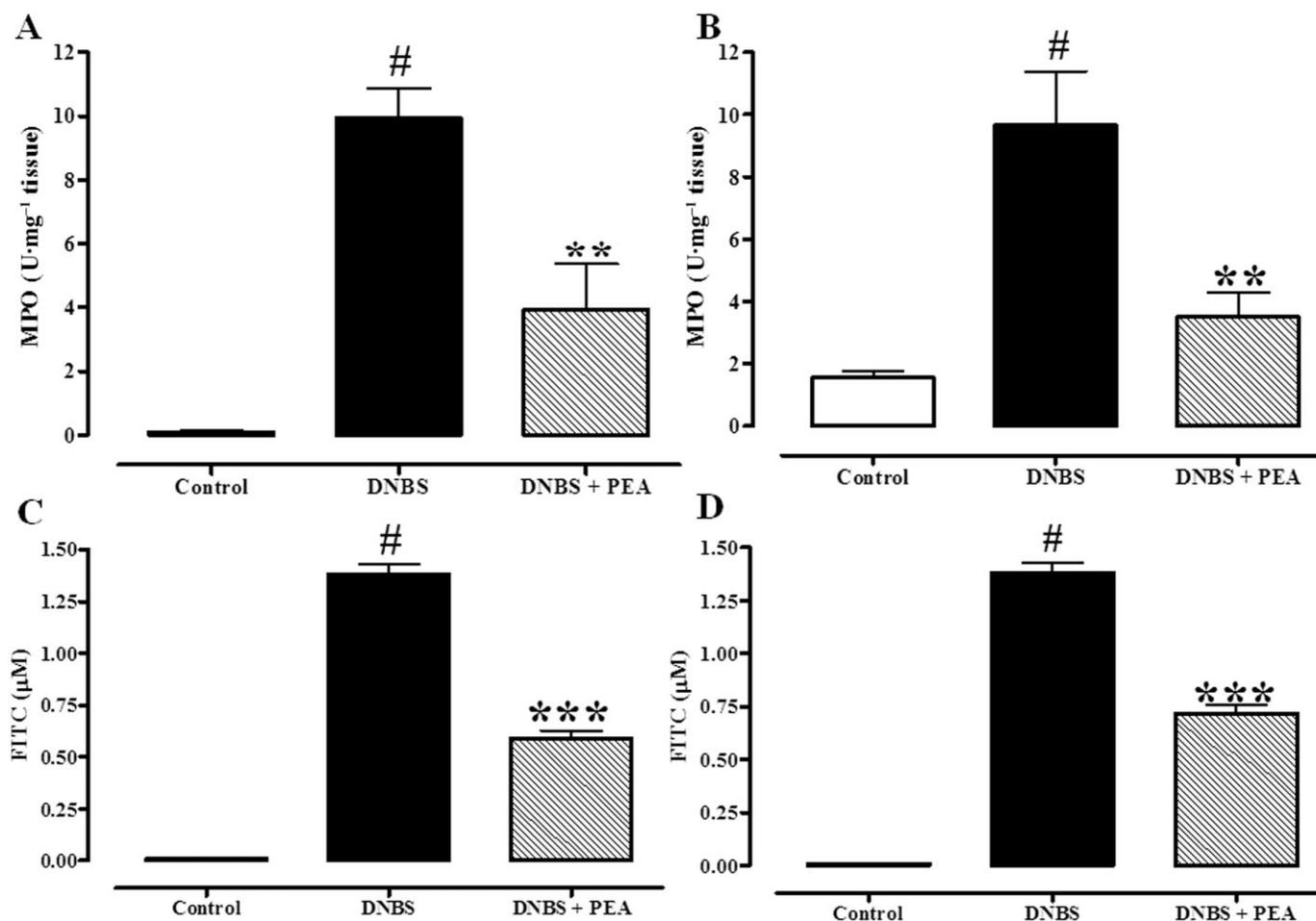


Figure 3

Inhibitory effect of PEA on MPO (a marker of intestinal inflammation) activity (A,B) and on serum FITC-dextran concentration (a measure of intestinal permeability; C,D) in DNBS-induced colitis in mice. Permeability and MPO activity were measured on colonic tissues 3 days after vehicle or DNBS administration. PEA [1 mg·kg⁻¹, i.p. (A,C) or p.o. (B,D)] was administered once a day for three consecutive days starting 24 h after the inflammatory insult (therapeutic protocol). Bars are mean ± SEM of four to five mice for each experimental group. #*P* < 0.001 versus control; ***P* < 0.01 and ****P* < 0.001 versus DNBS alone.

restricted to the two-thirds of the mucosa (i.e. the mature superficial cells were not in a proliferative state) (Figure 4E and F).

DNBS did not change mRNA expression of enzymes involved in PEA metabolism

In this set of experiments, we verified if changes in PEA levels were accompanied by variations in the mRNA expression of enzymes involved in PEA metabolism. Compared with control mice, DNBS administration did not significantly change the mRNA expression of GDE1, NAPE-PLD (two enzymes involved in PEA biosynthesis), FAAH and NAAA (two enzymes involved in PEA degradation) (Figure 5).

DNBS increased colonic PEA levels

Isotope dilution LC-MS analysis of lipid extracts from the colon of control and DNBS-treated mice showed that the endogenous levels of PEA significantly increased (by 2.5-fold) after DNBS administration, compared with control colons

(Figure 6A). OEA, another acylethanolamide chemically related to PEA, did not significantly change after DNBS administration (Figure 6B).

Oral PEA did not change the colonic levels of endocannabinoids

This set of experiments was performed because there is evidence in the literature that PEA can inhibit anandamide inactivation (Bisogno *et al.*, 1997; Di Marzo *et al.*, 2001) and thus indirectly activate endocannabinoid targets (i.e. CB receptors and TRPV1). As previously reported (Borrelli and Izzo, 2009; Borrelli *et al.*, 2009), the levels of both anandamide and 2-AG, compared with control colonic tissues, were significantly increased by treatment with DNBS (2.5-fold increase for anandamide and 2.1-fold increase for 2-AG) (Figure 6C and D). However, PEA (1 mg·kg⁻¹, p.o.) treatment did not affect OEA and endocannabinoid levels, either in control or DNBS-treated mice (Figure 6B–D). As expected, mice receiving p.o. PEA showed higher colonic levels of this

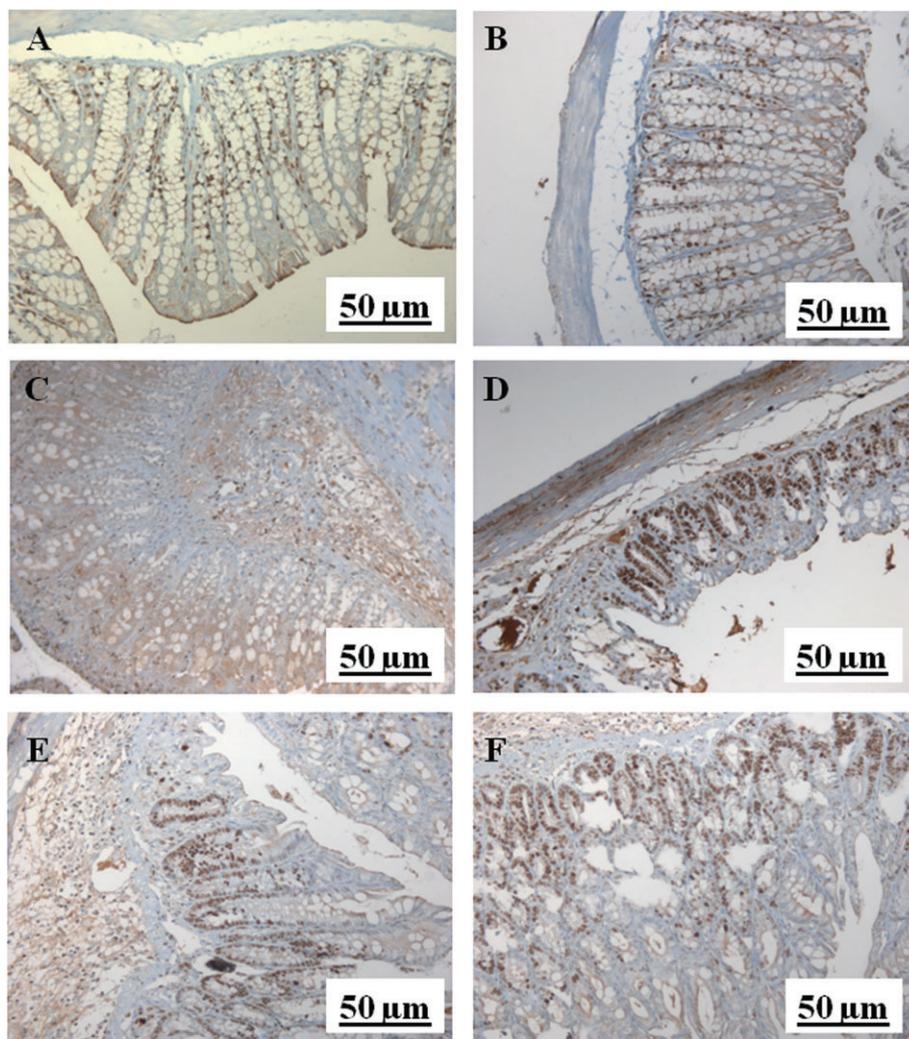


Figure 4

Different patterns of Ki-67 immunoreactivity in the colonic mucosa of control mice [i.e. mice receiving the vehicle used to dissolve PEA i.p. (A) or p.o. (B)], DNBS-treated mice receiving the vehicle used to dissolve PEA [i.p. (C) or p.o. (D)] and mice treated with DNBS plus PEA [i.p. (E) or p.o. (F)]. (A,B) Ki-67-immunopositive cells were localized to the lower part of the crypts. (C,D) Ki-67-immunopositive cells were extended to the superficial part of the crypts. (E,F) Ki-67-immunopositive cells were observed in the two-thirds of the mucosa, only. PEA ($1 \text{ mg}\cdot\text{kg}^{-1}$) was administered for three consecutive days starting 24 h after the inflammatory insult (therapeutic protocol). The Figure shows results representative of four experiments.

acylethanolamide, the effect being significant in DNBS-treated animals (Figure 6A).

Oral PEA affected colonic CB_1 , GPR55 and TRPV1 (but not CB_2 or $\text{PPAR}\alpha$) mRNA expression

In these experiments, we measured the mRNA expression of the receptors that are known to be directly or indirectly targeted by PEA. DNBS administration caused colonic down-regulation of GPR55 and TRPV1 mRNA expression, with no changes in CB (CB_1 and CB_2) receptors and $\text{PPAR}\alpha$ mRNA expression (Figure 7). PEA administration ($1 \text{ mg}\cdot\text{kg}^{-1}$, p.o.) counteracted (or partially counteracted) DNBS-induced GPR55 and TRPV1 down-regulation, while it up-regulated,

both in control and in DNBS-treated mice, CB_1 receptor mRNA expression (Figure 7).

The anti-inflammatory action of oral PEA involved CB_2 receptors, GPR55 and $\text{PPAR}\alpha$

In order to investigate the mode of action of PEA, we evaluated its pharmacological effects in the presence of rimona-bant (CB_1 receptor antagonist), AM630 (CB_2 receptor antagonist), ML-191 (GPR55 antagonist), GW6471 ($\text{PPAR}\alpha$ antagonist) and capsaizepine (TRPV1 channel antagonist). Results showed that AM630, ML-191 and GW6471 abolished or reduced the anti-inflammatory effects of PEA, as evaluated by the colon weight/colon length ratio (Figure 8A–E) and MPO activity (Figure 9A–E). The reversal by the antagonists

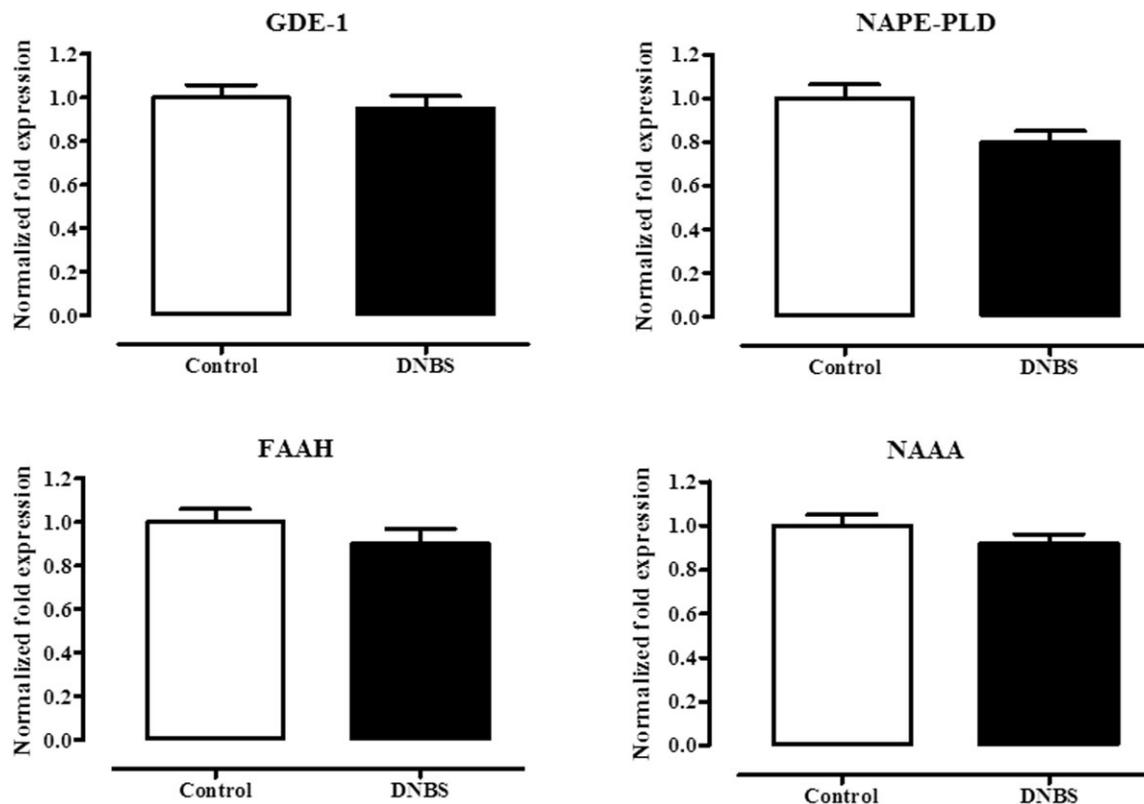


Figure 5

Relative mRNA expression of GDE1, NAPE-PLD, FAAH and NAAA in the colon of control and DNBS-treated mice. Tissues were analysed 3 days after vehicle or DNBS administration. RT-PCR analysis was performed as described in Methods. Results (expressed as fold expression, compared with control, as unity) are mean \pm SEM of six experiments. No significant effects of DNBS were found.

was significant, except for GW647, which significantly counteracted the effect of PEA on MPO activity (Figure 9D), but not on colon weight/colon length ratio (Figure 8D, $P = 0.06$). By contrast, capsazepine further increased the anti-inflammatory effect of PEA (Figure 8E and Figure 9E). The antagonists, at the doses used in the present investigation, did not modify, *per se*, DNBS-induced colitis. However, a higher dose of the GPR55 antagonist ML-191 (i.e. 1 mg·kg⁻¹), *per se*, slightly, but significantly, ameliorated DNBS-induced colitis, as revealed by the colon weight/colon length ratio [weight: length ratio (mg·cm⁻¹): control 23.7 \pm 3.1, DNBS 75.3 \pm 3.2#, DNBS + ML-191 63.4 \pm 3.7*; $n = 7$ for each experimental group. # $P < 0.001$ vs. control; * $P < 0.05$ vs. DNBS alone].

Discussion

PEA is a naturally occurring acylethanolamide considered to play protective and homeodynamic roles in the animal and vegetable kingdoms (Esposito and Cuzzocrea, 2013). The anti-inflammatory, analgesic and anti-convulsant properties of this amide are well established and are believed to be of potential therapeutic interest (Skaper and Facci, 2012). Although PEA-containing formulations are promoted for the treatment of intestinal complaints, the actions of this acylethanolamide in the digestive tract have been largely over-

looked to date. In the present study, we show that endogenous PEA is produced in the colon in response to an inflammatory insult and that exogenous-administered PEA exerts anti-inflammatory actions in the gut.

We have found that PEA, given *i.p.* or *p.o.*, reduced the colon weight/colon length ratio of the inflamed colonic tissue, which is considered a reliable and sensitive indicator of the severity and extent of the inflammatory response. PEA was effective when given after the inflammatory insult, suggesting a therapeutic beneficial effect. Furthermore, PEA was significantly more active when given *i.p.* than when given *p.o.*, a result that can be explained by the presence, in the digestive tract, of NAAA and other amidases able to metabolize PEA (Tsuboi *et al.*, 2007; Borrelli and Izzo, 2009). The effect of PEA was further supported by its ability to (i) reduce the decrease in body weight associated to DNBS administration, thus suggesting a favourable selective effect of this compound on overall mouse health in inflammatory conditions; (ii) decrease the histological signs of colon injury; (iii) lessen MPO activity, a well-established marker of neutrophil infiltration in mouse models of colitis (Krawisz *et al.*, 1984); (iv) partially restore the impaired intestinal permeability; and (v) limit the colonic diffusion of Ki-67, a useful marker for the evaluation of dysplasia in ulcerative colitis (Andersen *et al.*, 1998), as shown by immunohistochemistry, although we cannot exclude the possibility that

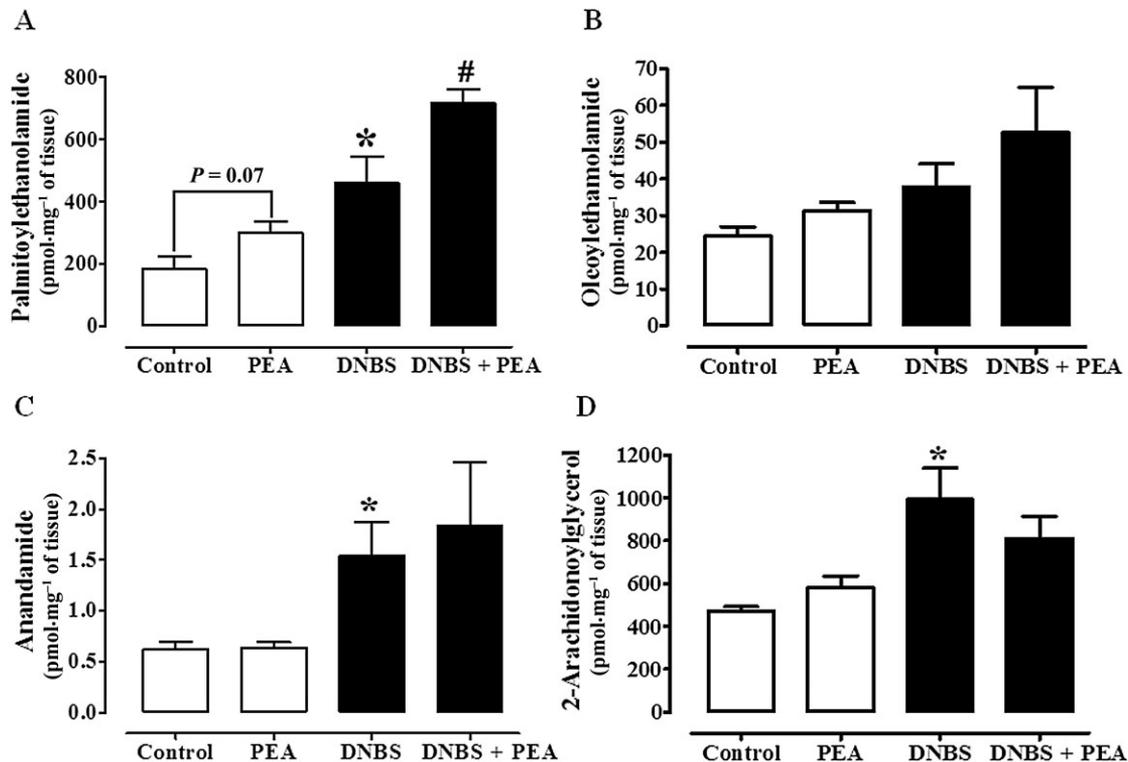


Figure 6

PEA (A), oleoylethanolamide (B), anandamide (C) and 2-arachidonoylglycerol (D) levels in colon of control mice and mice treated intracolonicly with the pro-inflammatory agent DNBS: effect of PEA. Tissues were analysed 3 days after vehicle or DNBS administration. PEA (1 mg·kg⁻¹) was administered p.o. once a day for three consecutive days starting 24 h after the inflammatory insult. Data are mean ± SEM of four to five mice. **P* < 0.05 versus control; #*P* < 0.05 versus DNBS alone.

PEA effect on Ki-67 could be an indirect effect related to its anti-inflammatory action. Previously, it has been shown that PEA (i.p.) significantly reduced intestinal transit in intestinal inflammatory (Capasso *et al.*, 2001) and post-inflammatory conditions (Capasso *et al.*, 2014) as well as the intestinal injury due to ischaemia reperfusion (Di Paola *et al.*, 2012). During the preparation of the present manuscript, others have shown that PEA *in vitro* exerted anti-inflammatory effects in colonic biopsies from IBD patients and ameliorated dextran sodium sulphate (DSS)-induced colitis when given i.p. to mice (Esposito *et al.*, 2014), an effect abolished by a PPAR α , but not a PPAR γ , antagonist. Compared with the results obtained by Esposito *et al.* (2014), we have used a different model of colitis, demonstrated the efficacy of PEA after p.o. administration and, importantly, broadened the investigation of PEA mode of action, by evaluating the potential involvement of endocannabinoids, CB receptors, TRPV1, GPR55 and PPAR α (results discussed below).

PEA is biosynthesized and metabolized in animal cells via a number of enzymes which, in many instances, are also involved in the biosynthesis and degradation of the endocannabinoid anandamide and of the anorectic mediator OEA (Ueda *et al.*, 2013). The presence of PEA in the gut is well documented and there is evidence that its intestinal levels may change in response to noxious stimuli (Borrelli and Izzo, 2009; Balvers *et al.*, 2013): for example, PEA has been shown

to increase in the duodenum of rats treated with methotrexate (a celiac disease-like model of intestinal atrophy) (D'Argenio *et al.*, 2007) and to decrease in the small intestine of mice treated with the pro-inflammatory agent croton oil (Capasso *et al.*, 2001; Izzo *et al.*, 2012). In the present study, we have observed a nearly threefold increase in colonic PEA levels in mice with experimental colitis, compared with control animals, with no changes in the mRNA expression of GDE1 and NAPE-PLD (two enzymes involved in PEA biosynthesis) as well as in the expression of NAAA and FAAH (two enzymes involved in PEA degradation). Relevant to this point is noteworthy that, although it is well established that FAAH inhibition results in intestinal anti-inflammatory effects (D'Argenio *et al.*, 2006; Storr *et al.*, 2008), there are no data in the literature concerning the effect of NAAA inhibitors in experimental models of colitis. NAAA inhibitors have been recently shown to attenuate tissue reactions to various pro-inflammatory stimuli, both *in vitro* and *in vivo* (Solorzano *et al.*, 2009; 2010; Li *et al.*, 2012). The increased levels of PEA in the present study was specific among the acylethanolamides, as the levels of OEA, which is co-released together with PEA, did not change significantly. Importantly, PEA levels are also elevated in patients with ulcerative colitis (Darmani *et al.*, 2005), thus confirming that the model used in the present study can be translated to the human condition, at least as far as the regulation of this mediator is concerned.

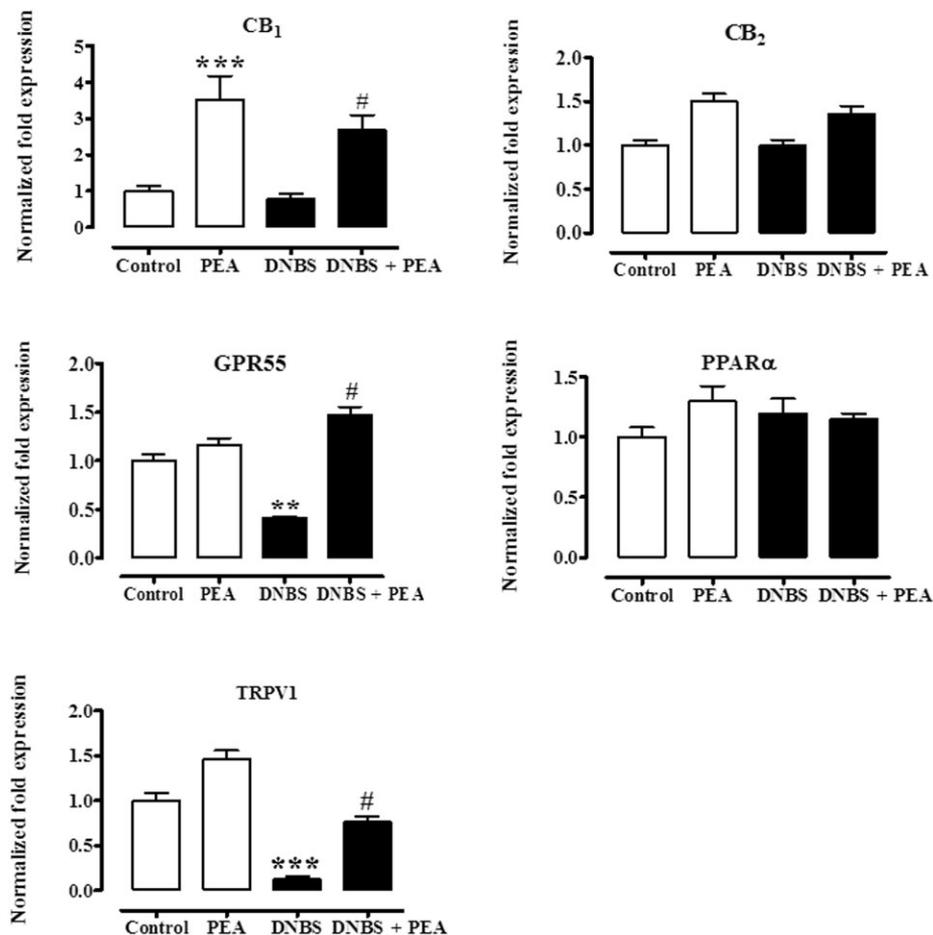


Figure 7

Relative mRNA expression of CB₁ receptors, CB₂ receptors, GPR55, PPAR α , and TRPV1 channels in the DNBS model of colitis: effect of PEA. (PEA, 1 mg·kg⁻¹) was administered p.o. once a day for three consecutive days starting 24 h after the inflammatory insult. Tissues were analysed 3 days after vehicle or DNBS administration. RT-PCR analysis was performed as described in Methods. Results (expressed as fold expression, compared with control equal to one) are mean \pm SEM of six experiments. ***P* < 0.01 and ****P* < 0.001 versus control; #*P* < 0.05 versus DNBS.

In mechanistic terms, there is evidence that PEA activates PPAR α (Lo Verme *et al.*, 2005; O'Sullivan and Kendall, 2010) and the orphan receptor GPR55 (Pertwee, 2007) and activates and desensitizes TRPV1 channels in sensory neurons (Ambrosino *et al.*, 2013). Additionally, PEA inhibits the inactivation of the endocannabinoid anandamide (Bisogno *et al.*, 1997; Di Marzo *et al.*, 2001) and potentiates the effect of anandamide at CB receptors or TRPV1 channels (the 'entourage effect') (De Petrocellis *et al.*, 2002; Smart *et al.*, 2002; Ho *et al.*, 2008). That said, a further step in our study was to evaluate the mRNA expression of such receptors in the colon of both control and DNBS-treated mice and, more importantly, to evaluate the pharmacological effect of PEA in the presence of the antagonists of the above-mentioned targets. Because the p.o. route of administration is desirable in IBD patients and because PEA is contained in over-the-counter preparations promoted for the treatment of intestinal complaints such as IBD, the experiments, which are discussed below, were performed by administering PEA p.o.

Persuasive evidence suggests that activation of both CB₁ and CB₂ receptors results in anti-inflammatory effect in the gut (Izzo and Camilleri, 2009; Alhouayek and Muccioli, 2012). In the present study, we have shown that the effect of PEA in the DNBS model of colitis was counteracted by a CB₂ receptor antagonist, suggesting an involvement of these receptors in PEA mode of action. Because PEA does not bind efficiently to CB₂ receptors (Lambert and Di Marzo, 1999), it is possible that PEA might activate such receptors via the so-called entourage effect, that is, the increase of the endocannabinoid levels and/or modulatory actions at CB receptors (De Petrocellis *et al.*, 2002; Smart *et al.*, 2002). Indeed, we found that DNBS administration caused an increase in endocannabinoid levels, which, however, were not further increased by PEA, probably because they were already maximally increased by the inflammatory insult. Pharmacological effects of PEA counteracted by CB₂ receptor antagonists have been previously documented (Calignano *et al.*, 1998; Farquhar-Smith *et al.*, 2002). Conversely, we found that,

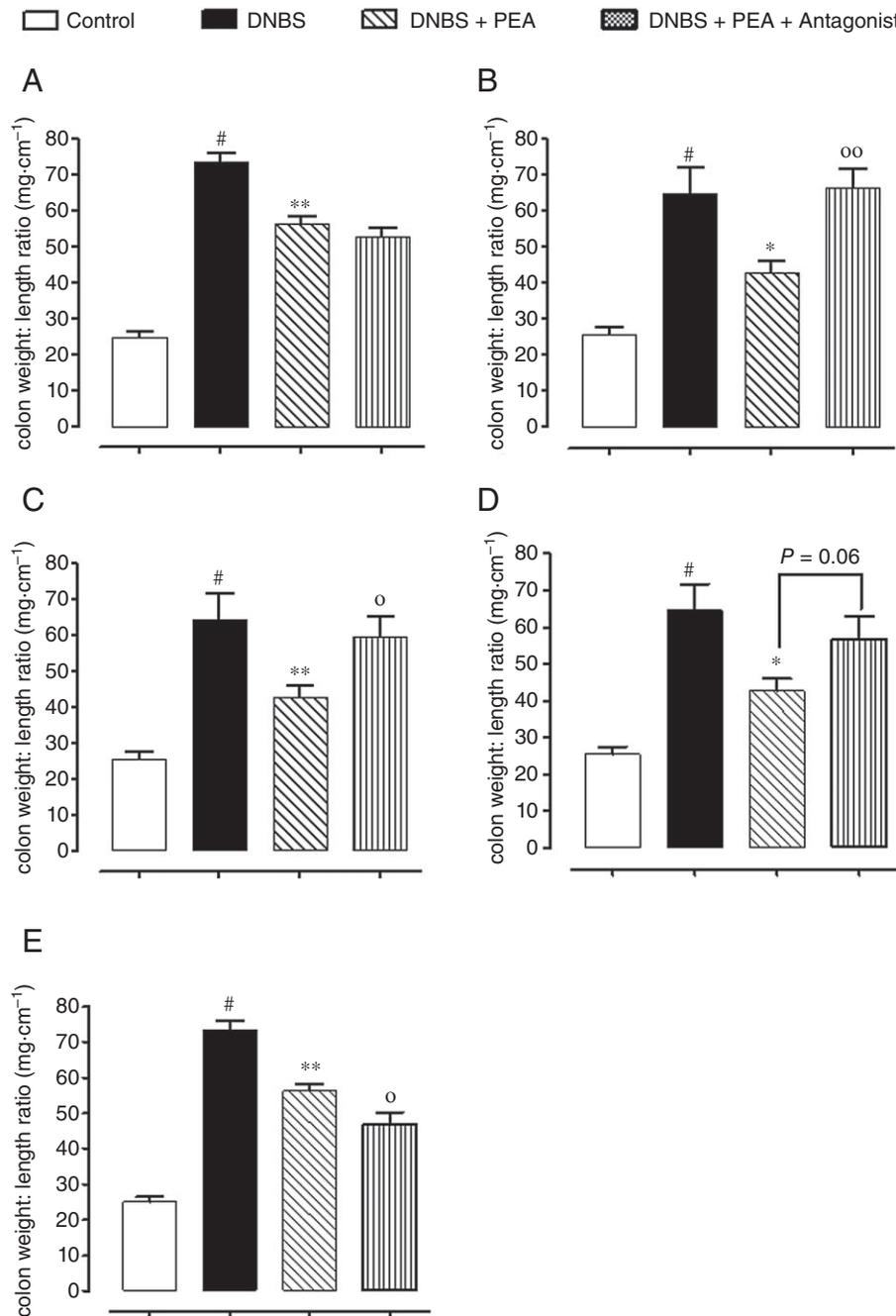


Figure 8

Effect of PEA alone or in the presence of the CB₁ receptor antagonist rimonabant (3 mg·kg⁻¹, i.p.) (A), the CB₂ receptor antagonist AM630 (10 mg·kg⁻¹, i.p.) (B), the GPR55 receptor antagonist ML-191 (0.5 mg·kg⁻¹, i.p.) (C), the PPAR α receptor antagonist GW6471 (1 mg·kg⁻¹, i.p.) (D), or the TRPV1 antagonist capsazepine (10 mg·kg⁻¹, i.p.) (E) on colon weight/colon length ratio in mice with experimental colitis induced by DNBS. PEA (1 mg·kg⁻¹, p.o.) was administered once a day for three consecutive days starting 24 h after the inflammatory insult. The antagonists were given 30 min before PEA administration. Tissues were analysed 3 days after vehicle or DNBS administration. #*P* < 0.01–0.001 versus control, **P* < 0.05 and ***P* < 0.01 versus DNBS, °*P* < 0.05 and °°*P* < 0.01 versus DNBS plus PEA (*n* = 6–8 mice for each experimental group).

although PEA up-regulated colonic CB₁ mRNA expression, a mechanism which is known to potentially exert anti-inflammatory effects in animal model of colitis (Di Marzo and Izzo, 2006), the CB₁ receptor antagonist rimonabant did not change the anti-inflammatory effect of PEA.

The orphan receptor GPR55 has been proposed to mediate some of the pharmacological actions of CBs (Pertwee, 2007; Ryberg *et al.*, 2007; Naderi *et al.*, 2012). Similar to CB₁ and CB₂ receptors, GPR55 is expressed throughout the rodent gastrointestinal tract (Schicho *et al.*,

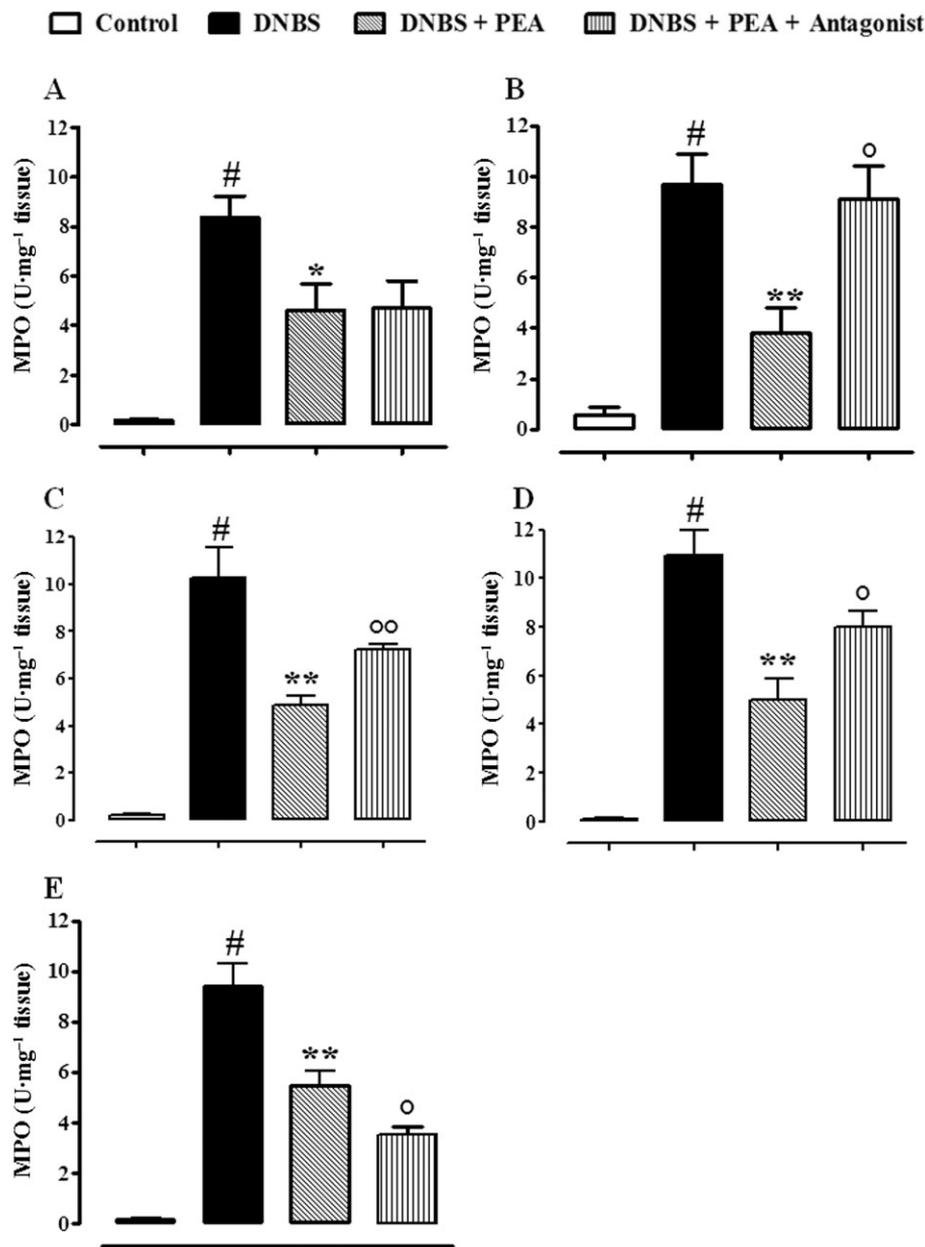


Figure 9

Effect of PEA alone or in the presence of the CB₁ receptor antagonist rimonabant (3 mg·kg⁻¹, i.p.) (A), the CB₂ receptor antagonist AM630 (10 mg·kg⁻¹, i.p.) (B), the GPR55 receptor antagonist ML-191 (0.5 mg·kg⁻¹, i.p.) (C), the PPAR α receptor antagonist GW6471 (1 mg·kg⁻¹, i.p.) (D) or the TRPV1 antagonist capsazepine (10 mg·kg⁻¹, i.p.) (E) on MPO activity in the murine model of colitis induced by DNBS. PEA (1 mg·kg⁻¹, p.o.) was administered once a day for three consecutive days starting 24 h after the inflammatory insult. The antagonists were given 30 min before PEA administration. MPO activity was measured on colonic tissues 3 days after vehicle or DNBS administration (therapeutic protocol). Bars are mean \pm SEM of four to five mice for each experimental group. #*P* < 0.01–0.001 versus control, **P* < 0.05 and ***P* < 0.01 versus DNBS, °*P* < 0.05 and °°*P* < 0.01 versus DNBS plus PEA.

2011; Schicho and Storr, 2012). In the present study, we have shown that the recently developed GPR55 antagonist ML-191 (Kotsikorou *et al.*, 2013), at a dose (i.e. 0.5 mg·kg⁻¹) which, *per se*, did not affect intestinal inflammation, reduced the beneficial effect of PEA in the DNBS model of colitis. The involvement of GPR55 was further supported by the observation that PEA restored GPR55 mRNA down-regulation caused by DNBS. Others have found that GPR55 mRNA expression decreased

in the model of pancreatitis induced by cerulein in mice (Li *et al.*, 2013) and increased in the intestine of rats challenged with LPS (Lin *et al.*, 2011).

It is well established that PPAR α has a role in controlling mucosal tissue homeostasis and PPAR α agonists ameliorate the experimental colitis induced by DNBS and DSS (Cuzzocrea *et al.*, 2004; Azuma *et al.*, 2010). In the present study, we have shown that the effect of PEA on MPO activity

in DNBS-treated animals was reduced by GW6471, a PPAR α receptor antagonist. PEA-induced changes in colon weight/colon length ratio were also reduced by GW6471, although the effect did not yield a full statistical significance ($P = 0.06$). Collectively such results suggest an involvement of PPAR α in PEA mode of action. Consistent with our results, Esposito *et al.* (2014) have recently demonstrated that MK866, another PPAR α antagonist, almost completely abolished the anti-inflammatory effect of i.p. PEA in the DSS model of murine colitis as well as in human ulcerative colitis biopsies.

There is also good evidence to suggest a pivotal role for TRPV1 in the development and maintenance of IBD (Kaneko and Szallasi, 2014). Increased TRPV1 immunoreactivity was reported in colonic biopsies taken from patients with IBD (Domotor *et al.*, 2005) and TRPV1 antagonists attenuate experimental colitis in mice (Kimball *et al.*, 2004). In the present study, we found that PEA significantly reduced TRPV1 mRNA down-regulation induced by DNBS and, more importantly, it exerted a stronger anti-inflammatory effect in the presence of the TRPV1 antagonist capsazepine, suggesting that this protein may negatively modulate the pharmacological activity of PEA, despite the fact that PEA itself is capable of desensitizing this channel (Ambrosino *et al.*, 2013). Consistent with such result, we have recently demonstrated that 5'-iodoresiniferatoxin, another TRPV1 antagonist, further increased the anti-prokinetic effect of PEA in a post-inflammatory murine model of accelerated gastrointestinal transit (Capasso *et al.*, 2014). Furthermore, de Novellis *et al.* (2012) have recently reported that PEA decreased the burst and increased the latency of tail flick-evoked onset of ON cell activity in the rostral ventromedial medulla and such effect was enhanced by a TRPV1 antagonist.

In conclusion, our study demonstrates that PEA, an endogenous lipid chemically related to the endocannabinoid anandamide – which is also contained in animal and vegetable foods – is produced in the gut in response to an inflammatory insult and exerts intestinal anti-inflammatory effects when administered i.p. and, more importantly, p.o. The protective effect of p.o. PEA, which might justify the use of PEA as a treatment for IBD, was associated to changes in TRPV1 channels, GPR55 and CB $_1$ receptor mRNA expression. Although we used pharmacological blockade rather than genetic deletion, our results clearly show that the effect of PEA is mediated by multiple targets, including CB $_2$ receptors, GPR55 and PPAR α and modulated by a TRPV1 channel antagonist.

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Author contributions

F. B. was responsible for acquisition, analysis and interpretation of data; conception and design; and redaction of the

manuscript. B. R. and P. O. conducted acquisition, analysis and interpretation of data, and critical reading of the manuscript. S. P., E. P., R. C., D. C. and G. B. carried out acquisition, analysis and interpretation of data. V. D. M. was responsible for conception and design, analysis and interpretation of data, and critical reading of the manuscript. A. A. I. performed conception and design, analysis and interpretation of data, and redaction of the manuscript.

Conflict of interest

Vincenzo Di Marzo is co-inventor of patents claiming the use of palmitoylethanolamide against inflammatory conditions, and receives research support from Epitech Italia S.r.l., which markets palmitoylethanolamide. Stefania Petrosino is an employee of Epitech Italia S.r.l. The other authors declare that they have no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.12907>

Table S1 Primer sequences.