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RESEARCH PAPER

The cannabinoid TRPA1 agonist cannabichromene inhibits nitric oxide production in macrophages and ameliorates murine colitis

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BACKGROUND AND PURPOSE
The non-psychotropic cannabinoid cannabichromene is known to activate the transient receptor potential ankyrin-type1 (TRPA1) and to inhibit endocannabinoid inactivation, both of which are involved in inflammatory processes. We examined here the effects of this phytocannabinoid on peritoneal macrophages and its efficacy in an experimental model of colitis.

EXPERIMENTAL APPROACH
Murine peritoneal macrophages were activated *in vitro* by LPS. Nitrite levels were measured using a fluorescent assay; inducible nitric oxide (iNOS), cyclooxygenase-2 (COX-2) and cannabinoid (CB_1 and CB_2) receptors were analysed by *RT*-PCR (and/or Western blot analysis); colitis was induced by dinitrobenzene sulphonic acid (DNBS). Endocannabinoid (anandamide and 2-arachidonoylglycerol), palmitoylethanolamide and oleoylethanolamide levels were measured by liquid chromatography-mass spectrometry. Colonic inflammation was assessed by evaluating the myeloperoxidase activity as well as by histology and immunohistochemistry.

KEY RESULTS
LPS caused a significant production of nitrites, associated to up-regulation of anandamide, iNOS, COX-2, CB_1 receptors and down-regulation of CB_2 receptors mRNA expression. Cannabichromene significantly reduced LPS-stimulated nitrite levels, and its effect was mimicked by cannabinoid receptor and TRPA1 agonists (carvacrol and cinnamaldehyde) and enhanced by CB_1 receptor antagonists. LPS-induced anandamide, iNOS, COX-2 and cannabinoid receptor changes were not significantly modified by cannabichromene, which, however, increased oleoylethanolamide levels. *In vivo*, cannabichromene ameliorated DNBS-induced colonic inflammation, as revealed by histology, immunohistochemistry and myeloperoxidase activity.

CONCLUSION AND IMPLICATIONS
Cannabichromene exerts anti-inflammatory actions in activated macrophages – with tonic CB_1 cannabinoid signalling being negatively coupled to this effect – and ameliorates experimental murine colitis.

Abbreviations

[^{35}S]GTPγS, [^{35}S] guanosine 5″-(gamma-thio) triphosphate; 2-AG, 2-arachidonoylglycerol; ACEA, arachidonyl-2′-chloroethylamide; AM251, *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AP-18, 4-(4-Chlorophenyl)-3-methyl-3-buten-2-one oxime; carvacrol, 5-isopropyl-2-methylphenol 2-Methyl-5-(1-methylethyl)-phenol; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; CBC, cannabichromene; CBD, cannabidiol; CGS 15943, 9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine; Cq, PCR quantitative-cycles; DAN, 2,3-diaminonaphtalene; DNBS, dinitrobenzene sulphonic acid; FITC, fluorescein isothiocyanate; GDP, guanosine diphosphate; HC-030031, 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl)acetamide; HEK-293, human embryonic kidney 293; IBD, inflammatory bowel disease; JWH133, (6a*R*,10a*R*)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*, *d*] pyran; MAGL, monoacylglycerol lipase; MOPS, 3-(N-morpholino)propanesulfonic acid; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide; NaPP, sodium diphosphate; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; rimonabant, 5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide; SR144528, [2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1H-pyrazole-3-carboxamide; TRPA1, transient receptor potential ankyrin-type1; Δ^9-THC, Δ^9-tetrahydrocannabinol

Introduction

Preparations derived from *Cannabis sativa* have been used medicinally to treat inflammatory conditions since the earliest written records on pharmacobotany (Zurier, 2003). For example, *Cannabis* was reported to be helpful against rheumatisms about 4000 years ago in the ancient China (Zurier, 2003). The empirical traditional uses of *Cannabis* encountered scientific evidence nearly 40 years ago, when it was demonstrated that a crude *Cannabis* extract exerted anti-inflammatory actions in the carrageenan-induced paw oedema model of acute inflammation in rats (Sofia *et al.*, 1974). The anti-inflammatory action of *Cannabis* was attributed to Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychotropic ingredient of the plant *Cannabis*, which activates both cannabinoid type 1 and 2 (CB_1 and CB_2) receptors, to cannabidiol (CBD), the main non-psychotropic component which does not efficiently activate cannabinoid receptors, and to cannabinol, a weak agonist of CB_1 and CB_2 receptors (Burstein and Zurier, 2009). More recently, Δ^9-THC and CBD were shown to be beneficial in experimental models of chronic inflammation such as arthritis (Malfait *et al.*, 2000; Cox and Welch, 2004) and have been administered as an oromucosal spray to patients with rheumatoid arthritis (Blake *et al.*, 2006).

In addition to Δ^9-THC, CBD and cannabinol, the plant *C. sativa* contains many other cannabinoids which could theoretically contribute to the anti-inflammatory effects of *Cannabis* preparations (Izzo *et al.*, 2009; Russo, 2011). One of such compounds is cannabichromene (CBC), the isolation and structure elucidation of which was reported in 1966 (Izzo *et al.*, 2009). CBC is one of four major cannabinoids in *C. sativa* and it is known to be abundant in high-grade drug-type marijuana, with little or no CBD (Holley *et al.*, 1975). CBC represents 0.3% of the constituents from confiscated *Cannabis* preparations in the USA (Mehmedic *et al.*, 2010). Despite the relative abundance of this phytocannabinoid, its pharmacological activity has been hardly investigated at all. Of relevance to the present study, CBC was shown to reduce carrageenan- and LPS-induced paw oedema in rodents (Wirth *et al.*, 1980; Turner and Elsohly, 1981; DeLong *et al.*, 2010). Pharmacodynamic studies have shown that CBC is an inhibitor of endocannabinoid cellular reuptake (Ligresti *et al.*, 2006), a weak inhibitor of monoacylglycerol lipase (MAGL, i.e. the main enzyme involved in the inactivation of the endocannabinoid 2-arachydonoylglycerol) and a potent activator of transient receptor potential (TRP) ankyrin 1-type (TRPA1) channels (De Petrocellis *et al.*, 2008; De Petrocellis *et al.*, 2012). Both endocannabinoids and TRPA1 are known to be involved in inflammatory processes (McMahon and Wood, 2006; Burstein and Zurier, 2009).

Given the traditional use of *Cannabis* preparations in treating inflammatory conditions and in the light of the consideration that TRPA1 and endocannabinoids play an important role in inflammation, we have here evaluated the effect of CBC on activated macrophages. In addition, since macrophages play a pivotal role in inflammatory bowel disease (IBD) (Yoshino *et al.*, 2010) and moreover, *Cannabis* preparations exert beneficial effects in IBD patients (Lahat *et al.*, 2012), we also evaluated the effect of CBC in a murine colitis model.

Methods

Animals

Male ICR mice (Harlan Laboratories, S. Pietro al Natisone, Italy), weighing 28–32 g, were used after 1-week acclimation period (temperature 23 ± 2°C and humidity 60%). Mice were fed *ad libitum* with standard food, except for the 24-h period immediately preceding the administration of dinitrobenzene sulphonic acid (DNBS). All animal procedures were in conformity 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).

Thioglycollate-elicited mouse peritoneal macrophages

Peritoneal macrophages were obtained from mice as previously described by Aviello *et al*. (2011). Briefly, to evoke the production of peritoneal exudates rich in macrophages, mice were injected intraperitoneally with 1 mL of 10% w/v sterile thioglycollate medium (Sigma-Aldrich, Milan, Italy). After 4 days, mice were killed and the peritoneal macrophages were collected and seeded in appropriate plates for performing *in vitro* experiments (Aviello *et al*., 2011).

Cell culture and inflammatory insult

Peritoneal macrophages were cultured in DMEM supplemented with 10% FBS. The inflammatory response in peritoneal macrophages was induced by LPS from *Escherichia coli* serotype O111 : B4 (1 $\mu g \cdot mL^{-1}$). The acute inflammatory response in macrophages required an LPS incubation time of 18 h (Aviello *et al*., 2011).

Cytotoxicity

Cell respiration was assessed by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. After incubation with the tested compounds for 24 h, macrophages (1×10^5 cells per well seeded in a 96-well plate) were incubated with MTT (250 $\mu g \cdot mL^{-1}$) for 1 h. After solubilization in dimethyl sulfoxide (DMSO), the extent of reduction of MTT to formazan was quantitated by measuring the optical density at 490 nm (iMark™ Microplate Absorbance Reader, Bio-Rad, Milan, Italy). Treatments were compared with a reference cytotoxic drug (DMSO 20% v/v). Results are expressed as a percentage of the corresponding controls (without treatment).

Nitrite measurement and pharmacological treatment in vitro

Nitrites, stable metabolites of NO, were measured in macrophages medium as previously described (Aviello *et al*., 2011). Macrophages (5×10^5 cells *per* well seeded in a 24-well plate) were incubated with CBC (0.001–1 μM) for 30 min and subsequently with LPS (1 $\mu g \cdot mL^{-1}$) for 18 h. After reduction of nitrates to nitrites by cadmium, cell supernatants were incubated with 2,3-diaminonaphtalene (DAN) (50 $\mu g \cdot mL^{-1}$) for 7 min. After stopping the reaction with 2.8 N NaOH, nitrite levels were measured using a fluorescent microplate reader (LS55 Luminescence Spectrometer; PerkinElmer Life Sciences, Cambridge, UK; excitation–emission wavelengths of 365–450 nm).

In a subsequent set of experiments, cannabinoid receptor antagonists [0.1 μM 5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide (rimonabant), 1 μM *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) and 0.1 μM N-[(1S)-endo-1,3,3-trimethylbicyclo [2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1H-pyrazole-3-carboxamide (SR144528) or a non-selective adenosine receptors antagonist [0.1 μM 9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine (CGS 15943)] were incubated 30 min before CBC (1 μM). In some experiments, cells were also treated with arachidonyl-2′-chloroethylamide (ACEA) (0.001–0.1 μM, CB_1 receptor agonists), (6a*R*,10a*R*)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*, *d*]pyran (JWH133) (0.001–0.1 μM, CB_2 receptor agonist), 5-isopropyl-2-methylphenol 2-Methyl-5-(1-methylethyl)-phenol (carvacrol) (0.001–0.1 M, TRPA1 agonist), (2*E*)-3-phenylprop-2-enal (cinnamaldehyde) (0.001–0.1 μM, TRPA1 agonist), 4-(4-Chlorophenyl)-3-methyl-3-buten-2-one oxime (AP-18) (10 and 20 μM, TRPA1 antagonists) and 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl) acetamide (HC-030031) (10 and 20 μM, TRPA1 antagonists), all incubated 30 min before LPS stimulation.

Western blot analysis

Cell lysates were separated on SDS polyacrylamide gel, and transferred to a nitrocellulose membrane (Protran®, Protran Nitrocellulose Transfer Membrane Schleicher & Schuell Bioscience, Germany) using a Bio-Rad Transblot as previously reported in detail (Aviello *et al*., 2010). Membranes were incubated with mouse anti-inducible nitric oxide (iNOS) and anti-cyclooxygenase-2 (COX-2) (BD Biosciences from Becton Dickinson, Buccinasco, Italy), and subsequently with mouse anti-peroxidase-conjugated goat IgG (Jackson ImmunoResearch from LiStarFish, Milan, Italy). The signals were visualized by enhanced chemiluminescence using ImageQuant 400 equipped with software ImageQuant Capture (GE Healthcare, Milan, Italy) and analysed using Quantity One Software version 4.6.3. The membranes were contextually probed with mouse monoclonal anti β-actin (Sigma-Aldrich), to normalize the results.

Interleukin-1β, interferon-γ and interleukin-10 levels

IL-1β, IFN-γ and IL-10 levels in cell medium after 18-h exposure to CBC 1 μM followed by LPS (1 $\mu g \cdot mL^{-1}$) were quantified using commercial ELISA kits (R&D Systems, from Space Import Export, Milan, Italy) according to the manufacturer's instructions.

Identification and quantification of endocannabinoids (anandamide and 2-AG) and related molecules

Endocannabinoid [(anandamide and 2-arachidonoylglycerol (2-AG)], palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) levels were measured in peritoneal macrophages (treated or not with LPS, 1 $\mu g \cdot mL^{-1}$ for 18 h). CBC (1 μM) was added 30 min before LPS challenge. Cells were harvested in 70% methanol before cell processing, subsequently extracted, purified and analysed by isotope dilution liquid chromatography-atmospheric pressure-chemical ionization mass spectrometry as previously described (Aviello *et al*., 2012).

Quantitative (real-time) RT-PCR analysis

Peritoneal macrophages (treated or not with CBC, 1 μM, 30 min before LPS) were collected and homogenized in 1.0 mL of Trizol® (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted according to the manufacturer's recommendations and further purified and DNA digested by the Micro RNA purification system (Invitrogen). Total RNA eluted from spin cartridge was UV-quantified by a Bio-Photometer® (Eppen-

dorf, Santa Clara, CA, USA), and purity of RNA samples was evaluated by the RNA-6000-Nano® microchip assay using a 2100 Bioanalyzer® equipped with a 2100 Expert Software® (Agilent, Santa Clara, CA, USA) following the manufacturer's instructions. For all samples tested, the RNA integrity number was greater than 8 relative to a 0–10 scale. One microgram of total RNA, as evaluated by the 2100 Bioanalyzer. Retro transcription, primer design and qPCR were performed as previously reported in detail (Grimaldi *et al.*, 2009). The amplification profile (10 ng of cDNA for assay) consisted of an initial denaturation of 2 min at 95°C and 40 cycles of 10 s at 96°C, annealing for 15 s at the optimal PCR annealing temperature and elongation for 25 s at 68°C. Assays were performed in quadruplicate, relative normalized expression was evaluated as previously described (Di Marzo *et al.*, 2008).

Radioligand assays: cell lines

Chinese hamster ovarian (CHO) cells, stably transfected with complementary DNA encoding human cannabinoid CB_1 receptors, were cultured in Eagle's medium nutrient mixture F-12 Ham supplemented with 1 mM L-glutamine, 10% v/v FBS and 0.6% penicillin-streptomycin together with geneticin (600 $\mu g \cdot mL^{-1}$).

Radioligand assays: membrane preparation

Binding assays with [^{35}S]GTPγS were performed with CB_1-CHO cell membranes (Brown *et al.*, 2010). The cells were removed from flasks by scraping and then frozen as pellets at –20°C until required. Before use in a radioligand binding assay, cells were defrosted, diluted in Tris-buffer (50 mM Tris-HCl, 50 mM Tris-Base) and homogenized. Protein assays were performed using a Bio-Rad DC kit (Hercules, CA, USA).

Radioligand assays: [^{35}S]GTPγS binding assays

Measurement of agonist-stimulated [^{35}S]GTPγS binding to cannabinoid CB_1 receptors was described previously (Brown *et al.*, 2010). The assays were carried out with GTPγS binding buffer (50 mM Tris-HCl, 50 mM Tris-Base, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM DTT and 0.1% bovine serum albumin) in the presence of [^{35}S]GTPγS and guanosine diphosphate (GDP), in a final volume of 500 µL. Binding was initiated by the addition of [^{35}S]GTPγS to the wells. Non-specific binding was measured in the presence of 30 µM GTPγS. Cannabinoid receptor antagonists, rimonabant or AM251, were incubated 30 min before CBC, at 30°C. Total incubation time was 60 min. The reaction was terminated by a rapid vacuum filtration method using Tris-binding buffer, as described previously, and the radioactivity was quantified by liquid scintillation spectrometry. In all the [^{35}S]GTPγS binding assays, we used 0.1 nM [^{35}S]GTPγS, 30 µM GDP and 33 µg *per* well of proteins.

Induction of experimental colitis and pharmacological treatment

Colitis was induced by the intracolonic administration of DNBS (Borrelli *et al.*, 2009). Briefly, mice were anaesthetized and DNBS (150 $mg \cdot kg^{-1}$) was inserted into the colon using a polyethylene catheter (1 mm in diameter) *via* the rectum (4.5 cm from the anus). Three days after DNBS 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 antimesenteric border, rinsed, weighed and length measured (in order to determine the colon weight/colon length *ratio*). For biochemical analyses, tissues were kept at –80°C until use, while for histological examination and immunohistochemistry tissues were fixed in 10% v/v formaldehyde. The dose of DNBS was selected on the basis of preliminary experiments showing a remarkable colonic damage associated to high reproducibility and low mortality for the 150 $mg \cdot kg^{-1}$ 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). Furthermore, previous studies have shown that 3 days after intracolonic DNBS administration in mice, the inflammatory response may be modulated by administration of cannabinoid drugs (Massa *et al.*, 2004; Borrelli *et al.*, 2009). CBC (0.1 and 1 $mg \cdot kg^{-1}$, i.p.) was injected for 2 consecutive days starting 24 h after DNBS administration. In some experiments, CBC (1 $mg \cdot kg^{-1}$, i.p.) was given as preventive treatment starting from 3 days before DNBS administration (once a day until the sacrifice).

Histology and immunohistochemistry

Histological and immunochemistry evaluations, performed 3 days after DNBS administration, was assessed on a segment of 1 cm of colon located 4 cm above the anal canal. After fixation for 24 h in saline 10% (v/v) formaldehyde, samples were dehydrated in graded ethanol and embedded in paraffin. Thereafter, 5-µm sections were deparaffinized with xylene, stained with hematoxylin–eosin, and observed in a DM 4000 B Leica microscope (Leica Microsystems, Milan, Italy). For microscopic scoring, we used a modified version of the scoring system reported by D'Argenio *et al.* (2006). Briefly, colon was scored considering (i) the submucosal infiltration (0, none; 1, mild; 2–3, moderate; 4–5 severe), (ii) the crypt abscesses (0, none, 1–2 rare; 3–5, diffuse) and (iii) the 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), were heated in a decloaking chamber at 125°C for 3 min and were cooled at room temperature for 20 min. After adding 3% hydrogen peroxide, sections were incubated for 10 min. After washing the sections with Tris-buffered saline Tween-20 (pH 7.6), they were stained with rabbit monoclonal antibody to Ki-67 (Ventana Medical systems, Tucson, AZ, USA). Briefly, each tissue section was incubated with primary antibody to Ki-67 (1:100) for 30 min at room temperature. The slides were washed three times with Tris-buffered saline Tween-20 and were incubated with secondary antibody for 30 min. After the slides were reacted with streptavidin for 20 min, the reaction was visualized by 3,3′-diaminobenzidine tetrahydrochloride for 5 min, and the slides were counterstained with Mayer's hematoxylin. The intensity and localization of immunoreactivities against the primary antibody used were examined on all sections with a microscope (Leica Microsystems).

Myeloperoxidase (MPO) activity in the colon

MPO activity was determined as previously described (Goldblum *et al.*, 1985). Full-thickness colons were homogenized in an appropriate lysis buffer [hexadecyltrimethylammonium bromide 0.5% in 3-(N-morpholino)propanesulfonic acid (MOPS) 10 mM] in *ratio* 50 mg tissue/1 mL MOPS. The samples were then centrifuged for 20 min at 15 000× *g* at 4°C. An aliquot of the supernatant was then incubated with NaPP (sodium phosphate buffer pH 5.5) e tetra-methylbenzidine 16 mM. After 5 min, H_2O_2 (9.8 M) in NaPP was added and the reaction stopped adding acetic acid. The rate of exchange 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 (Sigma-Aldrich). MPO activity was expressed as Units·mL^{-1}.

Intestinal permeability in the colon

Intestinal permeability was evaluated using a fluorescein isothiocyanate (FITC)-labelled-dextran method, as described previously (Osanai *et al.*, 2007). Briefly, 2 days after DNBS administration, mice were gavaged with 0.6 mg·g^{-1} body weight of FITC-conjugated dextran. One day later, blood was collected and the serum was immediately analysed for FITC-derived fluorescence using a fluorescent microplate reader with an excitation–emission wavelengths of 485–520 nm (LS55 Luminescence Spectrometer, PerkinElmer Instruments). Preliminary experiments showed that FITC-dextran was stable after 24 h from its preparation. Serial-diluted FICT-dextran was used to generate a standard curve.

Statistical analysis

Results are expressed as 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 Turkey–Kramer multiple comparisons test was used for analysis of multiple treatment means. *P*-values <0.05 were considered significant. Values obtained from the radioligand assays have been expressed as means and variability as SEM or as 95% confidence limits. Net agonist-stimulated [^{35}S]GTPγS binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values (obtained in the presence of agonist) as detailed elsewhere (Brizzi *et al.*, 2005). Values for EC_{50}, maximal effect (E_{max}) and SEM or 95% confidence limits of these values have been calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve (GraphPad Prism).

Materials

CBC, (purity by HPLC, 96.3%) was kindly supplied by GW Pharmaceuticals (Porton Down, Wiltshire, UK). Rimonabant and SR144528 were supplied by SANOFI Recherche, (Montpellier, France). ACEA, JWH133, AM251, AP-18, CGS15943 and HC-030031 were purchased from Tocris (Bristol, UK); LPS from *E. coli* serotype O111:B4, thioglycollate medium, cadmium, DAN, carvacrol, cinnamaldehyde, MTT, DNBS and FITC-conjugated dextran (molecular mass 3–5 kDa) were purchased from Sigma-Aldrich. All reagents for Western blot analysis and cell culture were obtained from Sigma-Aldrich, Bio-Rad Laboratories (Milan, Italy) and Microtech (Naples, Italy). For radioligand binding experiments, [^{35}S]GTPγS (1250 Ci·mmol^{-1}) was obtained from PerkinElmer Life Sciences, GTPγS from Roche Diagnostic (Indianapolis, IN), GDP from Sigma-Aldrich (UK). CBC was dissolved in ethanol (for *in vitro* experiments), in DMSO (for radioligand assays) or ethanol/Tween20/saline (1:1:8; for *in vivo* experiments). Rimonabant, SR144528, ACEA, JWH133, AM251, AP-18, CGS15943, HC-030031 were dissolved in DMSO. DNBS was dissolved in 50% ethanol (0.15 mL/mouse). The drug vehicles (0.01% ethanol *in vitro*; 0.1% DMSO for radioligand assays, 60 µL/mouse *in vivo*) had no significant effects on the responses under study.

Results

Cytotoxicity

Results are shown in Table 1. CBC, at concentration ranging from 0.001 to 1 µM, did not affect macrophage mitochondrial respiration after 24 h exposure. Similarly, TRP ligands such as carvacrol (0.001–0.1 µM), cinnamaldehyde (0.001–0.1 µM), AP-18 (10 and 20 µM) and HC-030031 (10 and 20 µM) as well as the CB_1 receptor agonist ACEA (0.001–0.1 µM), the CB_2 receptor agonist JWH133 (0.001–0.1 µM), the CB_1 receptor antagonists rimonabant (0.1 µM) and AM251 (1 µM), the CB_2 receptor antagonist SR144528 (0.1 µM) and the non-selective adenosine receptor antagonist CGS 15943 (0.1 µM) did not exert cytotoxic effects (Table 1).

CBC reduces nitrite levels in LPS-stimulated macrophages

In cells not treated with LPS, CBC (0.001–1 µM) did not modify basal nitrite levels [nitrite levels (nM) ± SEM: control 614.4 ± 31.5, CBC 0.001 µM 620.5 ± 32.1, CBC 0.01 µM 618.4 ± 24.6, CBC 0.1 µM 612.7 ± 29.6, CBC 1 µM 626.9 ± 36.2; $n = 12$]. LPS (1 µg·mL^{-1} for 18 h) administration caused a significant increase in nitrite production (Figure 1). A pretreatment with CBC (0.001–1 µM), 30 min before LPS, significantly reduced LPS-increased nitrite levels (Figure 1). CBC was also effective when given 15 h after LPS challenge (i.e. 3 h before nitrite assay) (see insert to Figure 1). No significant differences were found in CBC effect when the compound was given 30 min before LPS or 15 h after LPS (i.e. 3 h before the nitrite assay, see overlapping curves in the insert to Figure 1). Like CBC, the CB_1 receptor agonist ACEA (0.001–0.1 µM) and the CB_2 receptor agonist JWH133 (0.001–0.1 µM) reduced the production of nitrites stimulated by LPS when given 30 min before LPS [nitrite levels (nM) ± SEM: control 642.2 ± 51.6, LPS 1 µg·mL^{-1} 911.3 ± 42.4$^{\#}$, ACEA 0.001 µM 782.3 ± 12.0*, ACEA 0.01 µM 730.9 ± 20.4**, ACEA 0.1 µM 699.8 ± 18.1***; $n = 6$, $^{\#}P < 0.01$ vs. control, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. LPS alone. Control 842.0 ± 18.4, LPS 1 µg·mL^{-1} 1200 ± 55.3$^{\#}$, JWH133 0.001 µM 942.5 ± 70.7*, JWH133 0.01 µM 965.8 ± 58.7*, JWH133 0.1 µM 707.0 ± 83.6***; $n = 6$, $^{\#}P < 0.001$ vs. control, $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. LPS alone.].

Table 1

Effect of cannabichromene, TRPA1 ligands (carvacrol, cinnamaldehyde, AP-18 and HC-030031), cannabinoid receptor (CB_1 and CB_2) ligands (ACEA, JWH133, rimonabant, AM251, SR144528), adenosine receptor antagonist CGS 15943 and DMSO (used as a positive control) on cell viability in murine peritoneal macrophages. Cytotoxicity was evaluated using the MTT assay

Drugs	Concentration (μM)	Viability
Vehicle		99.93 ± 4.7
Cannabichromene	0.001	103.7 ± 8.0
	0.01	101.3 ± 4.4
	0.1	96.29 ± 2.9
	1	103.8 ± 3.6
Vehicle		100.1 ± 4.9
Carvacrol	0.001	98.8 ± 5.1
	0.01	96.2 ± 3.9
	0.1	90.6 ± 3.1
Vehicle		100.2 ± 2.5
Cinnamaldehyde	0.001	99.3 ± 2.1
	0.01	104.9 ± 2.7
	0.1	94.1 ± 2.4
Vehicle		99.9 ± 2.8
AP-18	10	99.4 ± 3.2
	20	103.7 ± 3.2
Vehicle		99.9 ± 2.8
HC-030031	10	100.5 ± 3.3
	20	107.8 ± 2.9
Vehicle		100.3 ± 2.2
ACEA	0.001	99.5 ± 2.9
	0.01	98.6 ± 1.5
	0.1	99.6 ± 3.1
Vehicle		99.9 ± 2.8
JWH133	0.001	101.6 ± 3.9
	0.01	97.9 ± 3.2
	0.1	100.7 ± 2.7
Vehicle		99.93 ± 3.4
Rimonabant	0.1	98.25 ± 1.8
Vehicle		99.93 ± 3.4
AM251	1	98.9 ± 2.7
Vehicle		100.0 ± 3.2
SR144528	0.1	94.9 ± 1.8
Vehicle		100.0 ± 4.1
CGS15943	0.1	95.8 ± 2.1
DMSO	20% v/v	24.50 ± 1.783***

Results are mean ± SEM of three experiments (in triplicates). ***$P < 0.001$ versus corresponding control (medium).

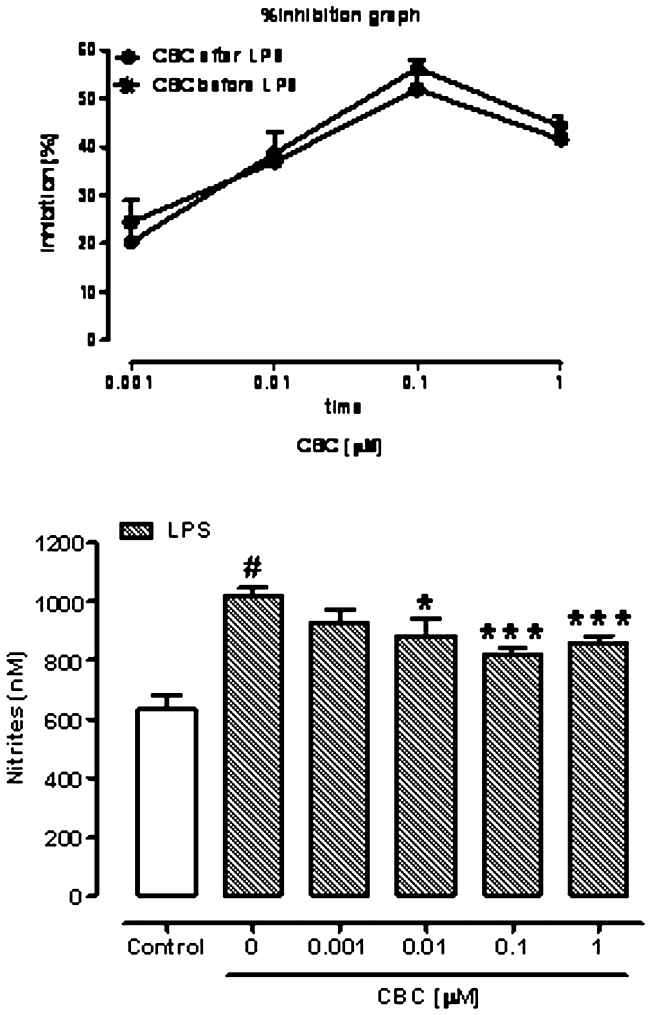

Figure 1

Inhibitory effect of cannabichromene on nitrite levels in the cell medium of murine peritoneal macrophages incubated with lipopolysaccharide (LPS, 1 μg·mL^{-1}) for 18 h. Cannabichromene (CBC, 0.001–1 μM) was added to the cell media 30 min before LPS challenge (i.e. 18.5 h before nitrites assay). Results are mean ± SEM of six experiments (in triplicates). $^{\#}P < 0.001$ versus control; $^{*}P < 0.05$ and $^{***}P < 0.001$ versus LPS alone. The insert (on top of the figure) shows the effect of CBC (expressed as percentage of inhibition of the corresponding control values, with the difference between LPS and control considered as 100%) when given 30 min before LPS (CBC before LPS) or 15 h after LPS (CBC after LPS). No statistically significant difference was observed between the two concentration–response curves reported in the insert.

CBC does not modify iNOS and COX-2 (mRNA and protein) levels in LPS-treated macrophages

In order to verify if the effect of CBC on the increased nitrite production was associated to changes in iNOS expression, we measured the mRNA and protein levels of this enzyme both by RT-PCR and by Western blot. LPS administration up-regulated iNOS mRNA and protein expression (Figure 2A,B). CBC (1 μM) incubated 30 min before LPS stimulation, did not modify LPS-induced changes in iNOS expression

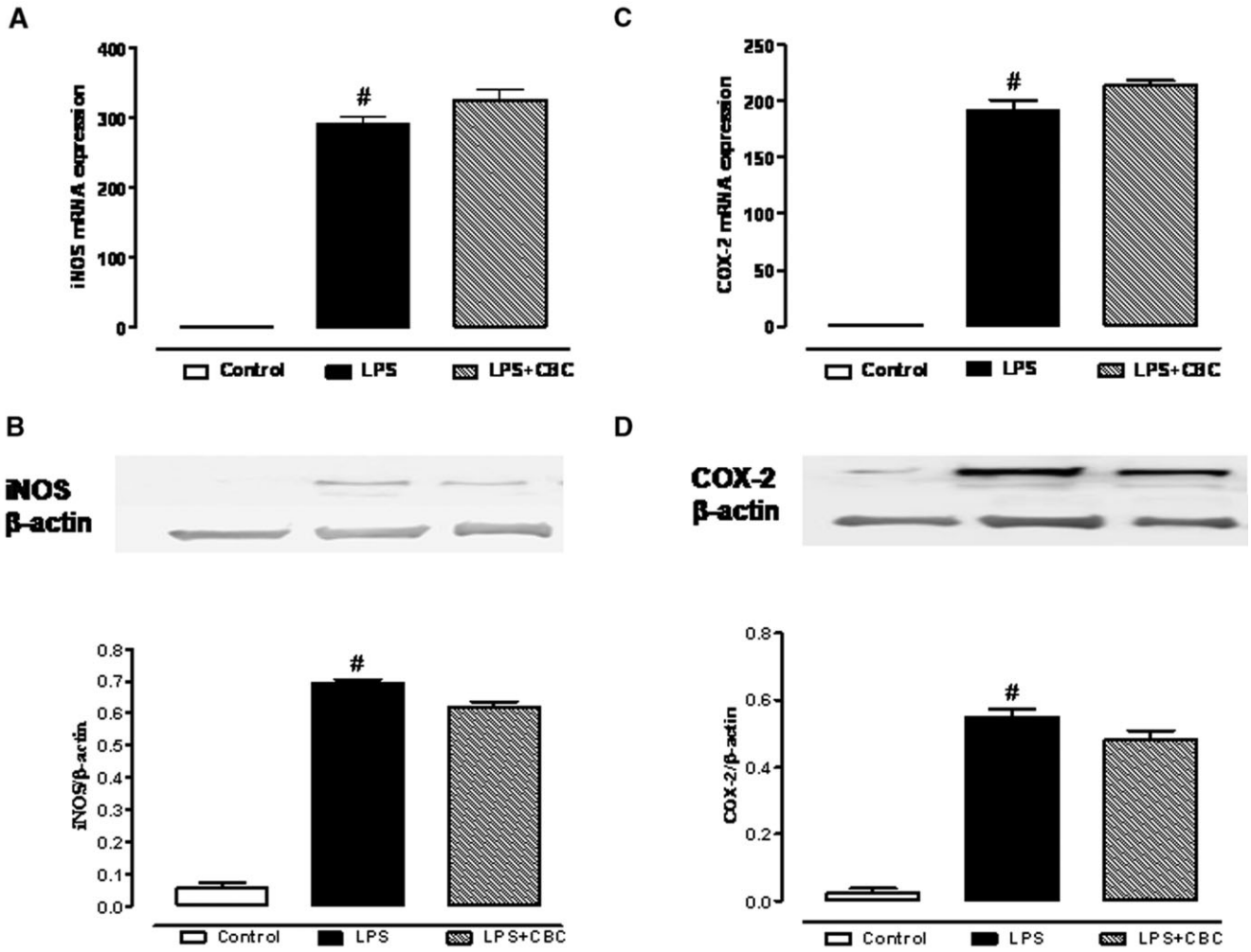

Figure 2

Inducible nitric oxide synthase (iNOS) (A, B) and cyclooxygenase-2 (COX-2) (C, D) mRNA and protein levels in cell lysates from macrophages incubated or not with lipopolysaccharide (LPS, 1 μg·mL^{-1}) for 18 h. mRNA expression was evaluated by RT-PCR. The expression levels, normalized with respect to the reference genes, were scaled to the expression value of the control, considered as 1. The means of the quantitative-cycles (Cq) for the control were: 26.00 and 25.58 for iNOS and COX-2 respectively. The reaction background was N/A (see text) at 40 reaction cycles. Protein expression was evaluated by Western blot analysis. Cannabichromene (CBC, 1 μM) was added to the cell media 30 min before LPS challenge. $^{\#}P < 0.001$ versus control (n = 4–5 experiments).

(Figure 2A,B). COX-2 is a key enzyme involved in the macrophages function. Similarly to iNOS, LPS administration caused up-regulation of COX-2 mRNA and protein expression (Figure 2C,D). CBC (1 μM) incubated 30 min before LPS stimulation, did not modify LPS-induced COX-2 up-regulation (Figure 2C,D).

CBC reduces IL-10 and INF-γ in LPS-treated macrophages

Interleukins and interferon-γ (INF-γ) are important cytokines involved in LPS-evoked responses in macrophages. The levels of IL-1β, *INF-γ* and IL-10 in macrophages medium were significantly increased after 18-h exposure to LPS (Figure 3A–C). A pretreatment with CBC (1 μM), incubated 30 min before LPS stimulation, significantly reduced INF-γ and IL-10 (but not IL-1β) levels in macrophages (Figure 3A–C).

The effect of CBC on nitrite production is modulated by selective CB_1 receptor antagonists

Because CBC can inhibit endocannabinoid inactivation (De Petrocellis *et al.*, 2011), in this set of experiments, we verified if CBC effect on nitrite production was reduced or counteracted by selective CB_1 and CB_2 receptor antagonists. We found that rimonabant (0.1 μM) and AM251 (1 μM) (two CB_1 receptor antagonists) not only did not counteract but, instead, significantly enhanced the inhibitory effect of CBC (1 μM) on nitrite production (Figure 4A,B). By contrast, the CB_2 receptor antagonist SR144528, at a concentration (0.1 μM) able to block the effect of the selective CB_2 receptor agonist JWH133 (0.1 μM) on nitrite production (data not shown) did not modify CBC (1 μM)-induced changes in nitrite production (Figure 4C). Rimonabant, AM251 and

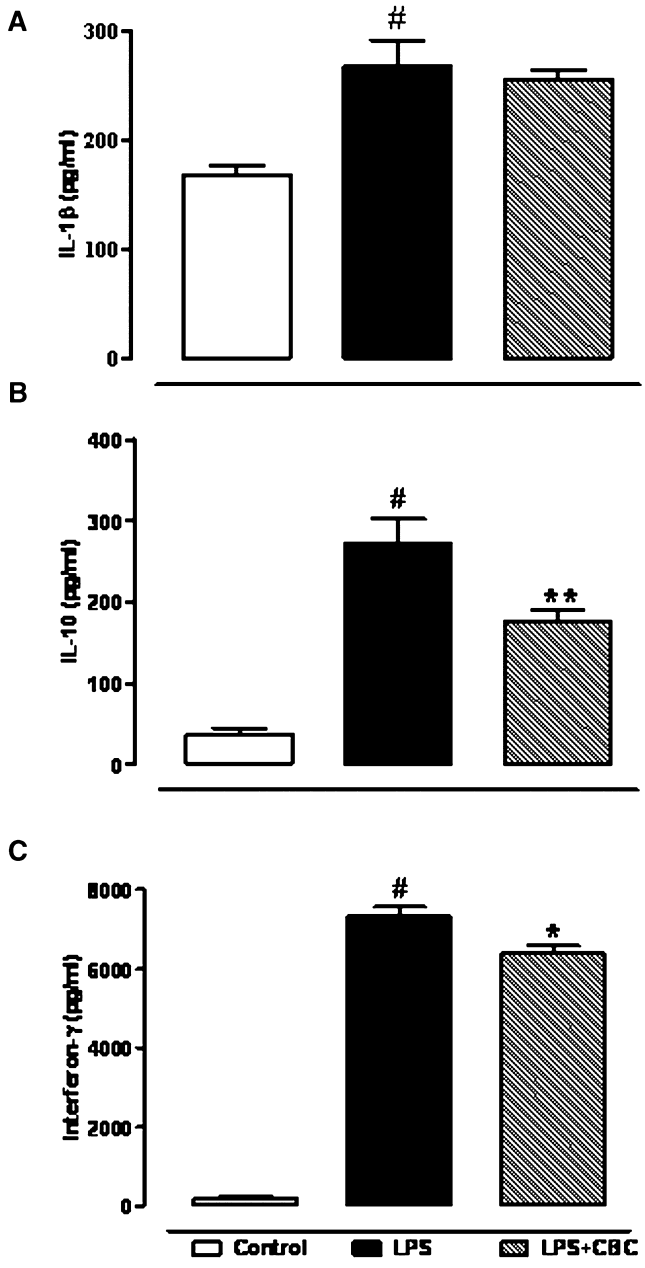

Figure 3

Effect of cannabichromene (CBC) on interleukin-1β (IL-1β) (A), interleukin-10 (IL-10) (B) and interferon-γ (C) levels detected in the cell media of macrophages incubated with lipopolysaccharide (LPS, 1 μg·mL^{-1}) for 18 h. CBC (1 μM) was added to the media 30 min before LPS challenge. Results are means ± SEM of four experiments (in quadruplicates). $^{\#}P < 0.001$ versus control, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus LPS.

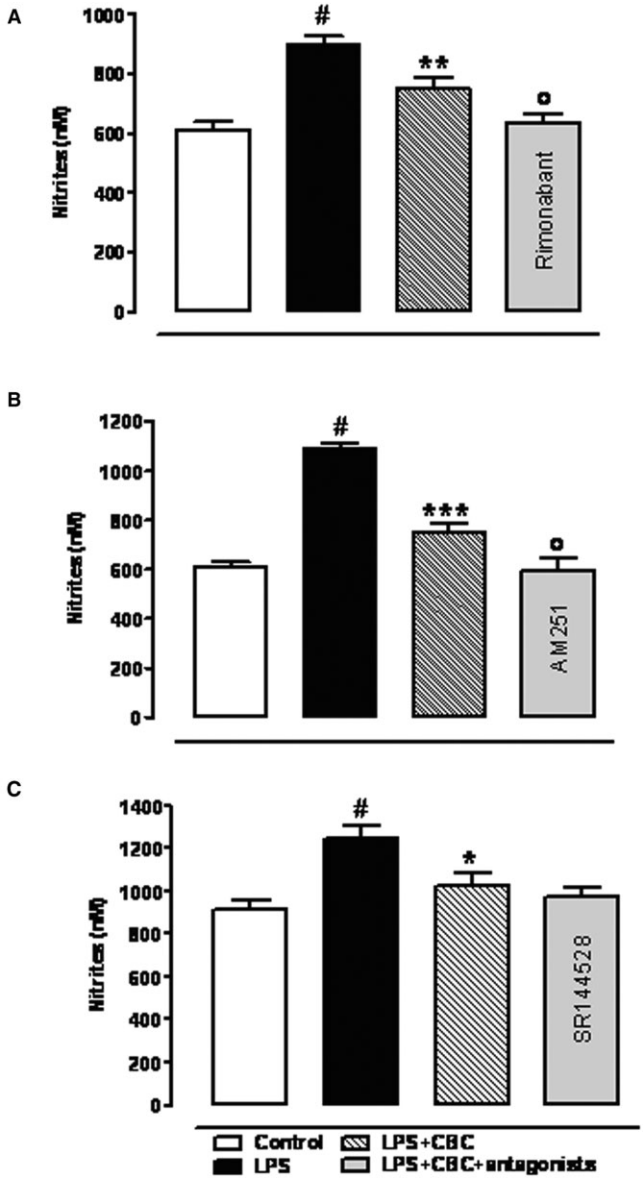

Figure 4

Effect of cannabichromene (CBC, 1 μM) alone and in presence of the cannabinoid CB_1 receptor antagonists rimonabant (0.1 μM) (A) and AM251 (1 μM) (B) as well as in the presence of the cannabinoid CB_2 receptor antagonist SR144528 (0.1 μM) (C) on nitrite levels in the cell medium of murine peritoneal macrophages incubated with lipopolysaccharide (LPS, 1 μg·mL^{-1}) for 18 h. The antagonists were added to the cell media 30 min before CBC exposure. LPS (1 μg·mL^{-1}for 18 h) was incubated 30 min after CBC. Results are means ± SEM of three experiments (in triplicates). $^{\#}P < 0.001$ versus control; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus LPS; $^{\circ}P < 0.05$ versus LPS + CBC.

SR144528, at the concentrations used, did not modify, *per se*, nitrite levels induced by LPS [nitrite levels (nM) ± SEM: control 611.9 ± 27.4, LPS 1 μg·mL^{-1} 899.1 ± 25.2$^{\#}$, rimonabant 0.1 μM 863.1 ± 24.8, AM251 1 μM 881.8 ± 21.5, SR144528 0.1 μM 917.1 ± 27.2; $n = 6$, $^{\#}P < 0.001$ vs. control.].

Next, using [^{35}S]GTPγS binding assays, we found that when tested at concentrations from 1 nM up to 1 μM, CBC did not display any significant ability to stimulate or inhibit [^{35}S]GTPγS binding to hCB_1-CHO cell membranes (data not shown). In contrast, using the same experimental conditions, we found that, when incubated by themselves, 1 μM AM251 and 0.1 μM rimonabant each induced, as expected, a marked inhibition of [^{35}S]GTPγS binding in this bioassay. When 1 μM CBC was added 30 min after 1 μM AM251 or 0.1 μM rimona-

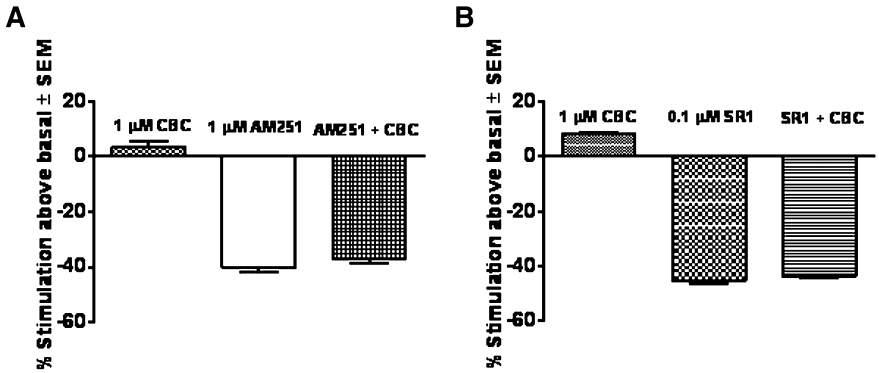

Figure 5

Effect of cannabichromene (CBC, 1 μM) alone and in combination with AM251 (1 μM, CB_1 receptor antagonist, A) or rimonabant (SR1, 0.1 μM, CB_1 receptor antagonist, B) on [^{35}S]GTPγS binding to hCB_1- CHO cell membranes (n = 12–16). CBC was added 30 min after the CB_1 antagonists. Symbols represent mean values ± SEM.

Table 2

Anandamide (AEA), 2-arachydonylglycerol (2-AG), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) levels in cell lysates from macrophages incubated or not with lipopolysaccharide (LPS, 1 μg·mL^{-1}) for 18 h: effect of cannabichromene (CBC, 1 μM, added alone to the cell media or 30 min before LPS challenge)

Drugs	AEA	2-AG	PEA	OEA
Vehicle	0.58 ± 0.13	102.1 ± 15.9	18.4 ± 2.9	9.52 ± 1.4
CBC	0.65 ± 0.22	121.8 ± 38.9	18.3 ± 5.5	7.7 ± 2.4
LPS	1.85 ± 0.55#	122.7 ± 25.9	25.6 ± 6.0	9.73 ± 2.4
LPS + CBC	1.5 ± 0.72	173.9 ± 23.0	33.9 ± 4.2	20.4 ± 2.9*

Results (pmol·mg^{-1} lipid) are mean ± SEM of three–six experiments. #P < 0.01 versus control; *P < 0.05 versus LPS.

bant, no significant change in E_{max} of either of these inverse agonists/antagonists was observed for their production of this inhibition (Figure 5A,B).

TRPA1 ligands reduce nitrite levels in LPS-treated macrophages

Because CBC can activate TRPA1 (De Petrocellis *et al.*, 2008; 2011), we verified if other TRPA1 agonists mimicked the effect of CBC on nitrite production in macrophages treated with LPS. Like CBC, carvacrol and cinnamaldehyde (incubated 30 min before LPS stimulation), both in the 0.001–0.1 μM range, reduced, in a concentration-dependent fashion, nitrite production induced by LPS (Figure 6A,B). However, AP-18 (10 and 20 μM) and HC-030031 (10 and 20 μM), two selective TRPA1 antagonists, inhibited nitrites production induced by LPS administration in macrophages (Figure 6C), suggesting that the effects of the agonists (and possibly CBC) could also be due to activation and subsequent desensitization of TRPA1.

CBC does not modify CB_1, CB_2 and TRPA1 mRNA alterations induced by LPS in macrophages

In contrast to CB_1 and CB_2 receptors mRNA, which were robustly expressed (see legend to Figure 7), TRPA1 mRNA was barely detectable in control macrophages (~35 PCR quantitative-cycles (Cq) vs. background; mouse colon TRPA1 positive control was detectable at 27 Cq). LPS (1 μg·mL^{-1}for 18 h) challenge caused up-regulation of CB_1 receptors, down-regulation of CB_2 receptors (Figure 7) and an even lower expression of TRPA1 mRNA, the expression of which resulted undetectable. CBC did not modify CB_1 and CB_2 (Figure 7) nor TRPA1 mRNA expression in LPS-treated macrophages.

CBC increases OEA (but not endocannabinoid) levels in LPS-treated macrophages

Table 2 reports the levels of endocannabinoids, PEA and OEA in murine peritoneal macrophages treated with LPS. The exposure to LPS (1 μg·mL^{-1}) for 18 h induced a significant increase in anandamide (but not 2-AG, PEA or OEA) levels. CBC (1 μM) did not change the levels of the endocannabinoids and PEA in control macrophages (i.e. not treated with LPS), nor in macrophages challenged with LPS (Table 2). By contrast, CBC significantly increased OEA levels in LPS-treated macrophages (Table 2).

The effect of CBC on nitrite production is not counteracted by a adenosine receptors antagonist

Because some of the pharmacological action of CBC have been shown to be counteracted by the adenosine receptors

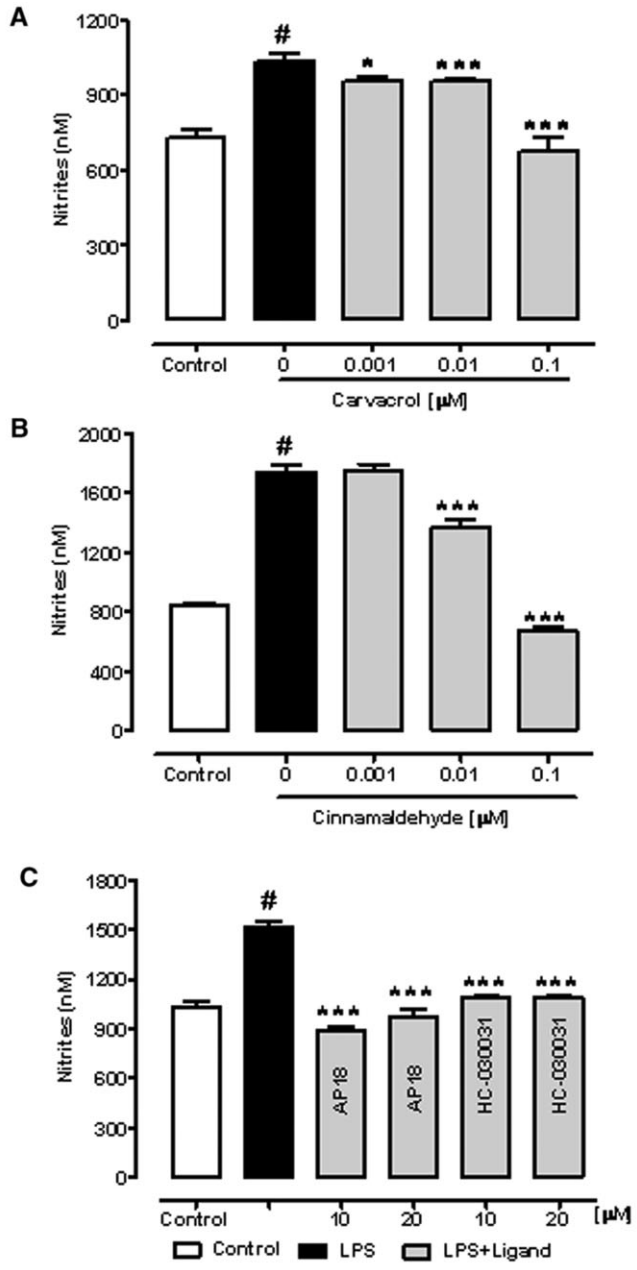

Figure 6

Inhibitory effect of the selective transient receptor potential ankyrin type 1 (TRPA1) agonists carvacrol (0.001–0.1 μM) and cinnamaldehyde (0.001–0.1 μM) (A, B) and of the selective TRPA1 antagonists AP18 (10–20 μM) and HC-030031 (HC, 10–20 μM) (C) on nitrite levels detected in the cell media of macrophages incubated with lipopolysaccharide (LPS, 1 μg·mL^{-1}) for 18 h. Both TRPA1 agonists and antagonists were added to the medium 30 min before LPS challenge. Results are mean ± SEM of two experiments (in triplicates). $^{\#}P < 0.001$ versus control; $^{*}P < 0.05$, and $^{***}P < 0.001$ versus LPS.

antagonist CGS 15943 (Maione *et al.*, 2011), we investigated the action of this antagonist on CBC-induced changes on nitrite production. We found that CGS 15943 (0.1 μM) did not modify CBC-induced reduction in nitrites levels in macrophages challenged with LPS [Nitrite levels (nM) ± SEM: control 809.3 ± 28.5, LPS 1 μg·mL^{-1} 998.7 ± 25.7$^{\#}$, CBC 0.1 μM 841.1 ± 12.9***, CBC 0.1 μM + CGS15943 0.1 μM 810.6 ± 31.3; $n = 12$, $^{\#}P < 0.001$ vs. control; $^{***}P < 0.001$ vs. LPS alone]. CGS 15943 (0.1 μM), *per se*, did not modify nitrite productions in LPS-treated macrophages (data not shown).

CBC ameliorates DNBS-induced colitis (colon weight/colon length ratio, intestinal permeability MPO activity, histology and immunohistochemistry)

Because macrophages play a pivotal role in inflammatory diseases, including IBD, we investigated the effect of this phytocannabinoid in an experimental model of colitis. DNBS administration caused a significant increase in colon weight/colon length *ratio* (Figure 8). CBC, at the doses of 0.1 and 1 mg·kg^{-1} [given intraperitoneally after the inflammatory insult], significantly reduced the effects of DNBS on colon weight/colon length *ratio*. The effect was significant for the dose of 1 mg·kg^{-1}. At the 1 mg·kg^{-1} dose, CBC significantly reduced DNBS-induced increase in intestinal permeability (Figure 9A) and MPO activity (Figure 9B). CBC (1 mg·kg^{-1}) exerted protective effects also when given before the inflammatory insult [colon weight/colon length *ratio* ± SEM: control 26.7 ± 1.2, DNBS 41.6 ± 1.9$^{\#}$, CBC (1 mg·kg^{-1}, i.p.) 30.6 ± 1.9**; $n = 9$ mice, $^{\#}P < 0.001$ vs. control; $^{**}P < 0.01$ vs. DNBS].

Histological analysis showed, in control mice, a normal appearance, with intact epithelium of the colonic mucosa (Figure 10A). In DNBS-treated mice, subtotal erosions of the mucosa, and diffuse lymphocyte infiltration involving the *muscularis mucosae* and the submucosa were observed (Figure 10B). CBC treatment (1 mg·kg^{-1}, given i.p. after DNBS) resulted in a regenerative area surrounding the residual focal erosions (Figure 10C).

Immunohistochemical analyses confirmed the beneficial effect of CBC on inflamed colonic mucosa. In control tissues, Ki-67 immunoreactivity revealed proliferative activity on the *fundus* of the foveole glands (Figure 11A). In the colon from DNBS-treated mice, total necrosis with Ki-67 immunoreactivity on inflammatory cells was observed (Figure 11B). CBC (1 mg·kg^{-1}, given i.p. after DNBS) reduced the effect of DNBS on cell proliferation, the mitotic activity being restricted to one half of the mucosa (Figure 11C).

Discussion

Preparations of *Cannabis* have been used since antiquity as medicinal agents to alleviate the symptoms of inflammation, including IBD (Zurier, 2003). The effect of Δ^9-THC and CBD, two main *Cannabis* constituents, on the inflammatory response is well established and their effect on inflammation has been extensively reviewed (Burstein and Zurier, 2009; Booz, 2011). However, the issue of whether other *Cannabis* constituents contribute to the anti-inflammatory effect of the plant is still under investigation. CBC has been shown to reduce the paw oedema induced by carrageenan or LPS in rodents (Turner and Elsohly, 1981; DeLong *et al.*, 2010) as well as to exert anti-inflammatory activity in the croton oil

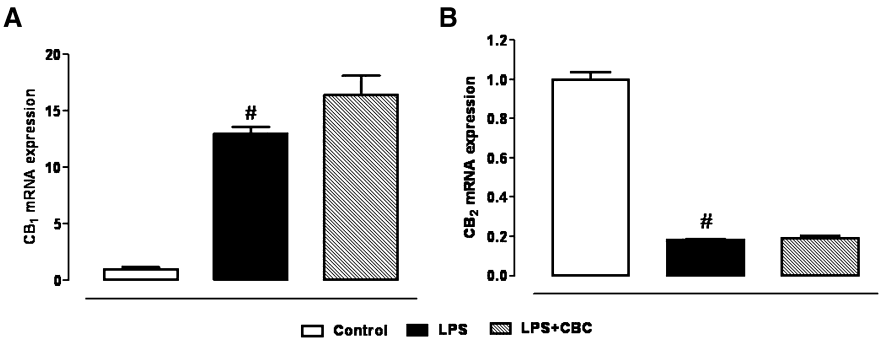

Figure 7

Relative mRNA expression of cannabinoid CB_1 receptor (A) and cannabinoid CB_2 receptor (B) in cell lysates from macrophages incubated or not with lipopolysaccharide (LPS, 1 μg·mL^{-1}) for 18 h: effect of cannabichromene (CBC, 1 μM, added to the cell media or 30 min before LPS challenge). The expression levels of mRNA, evaluated by qRT-PCR and normalized with respect to the reference genes, was scaled for all conditions to the expression value of the control, considered as 1. The means of the quantitative-cycles (Cq) for the control values were: 31.2 (CB_1 receptor) and 24.48 (CB_2 receptor). The reaction background was 37.30 Cq and 36.60 Cq for CB_1 receptor and CB_2 receptor, respectively, at 40 reaction cycles. $^{\#}P < 0.001$ versus control ($n = 4$).

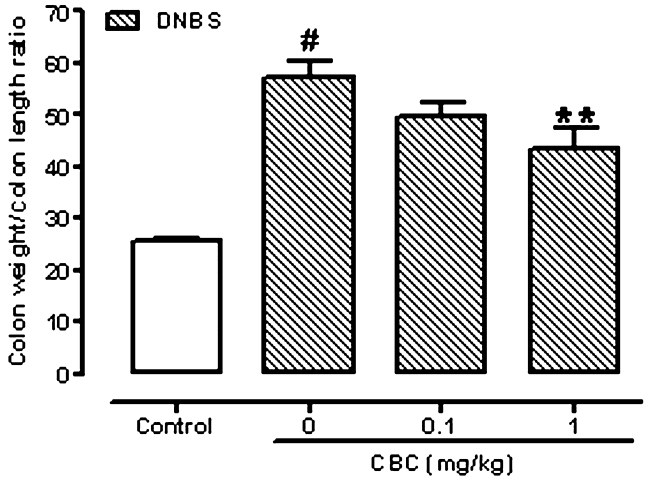

Figure 8

Dinitrobenzene sulfonic acid (DNBS)-induced colitis in mice. Colon weight/colon length *ratio* of colons from control and DNBS-treated mice in the presence or absence of cannabichromene (CBC). Tissues were analysed 3 days after vehicle or DNBS (150 mg·kg^{-1}, intracolonically) administration. CBC (0.1 and 1 mg·kg^{-1}) was administered (i.p.) once a day for 2 consecutive days starting 24-h after the inflammatory insult. Bars are mean ± SEM of 12–15 mice for each experimental group. $^{\#}P < 0.001$ versus control; $^{**}P < 0.01$ versus DNBS alone.

mouse ear dermatitis assay (Tubaro *et al.*, 2010). In the present study, we have demonstrated that CBC inhibits nitric oxide production in LPS-stimulated murine macrophages and ameliorates experimental colitis in mice.

CBC inhibits nitric oxide production in macrophages

Macrophages play a central role in the inflammatory process. Stimulation of murine macrophages by LPS results in the expression of iNOS, which catalyses the production of large amounts of NO, a gaseous substance which is proinflammatory when produced in excess (Moncada *et al.*, 1991). We have found here that CBC reduced the levels of nitrites, the stable metabolites of nitric oxide. It is unlikely that CBC affects the processes linked to the induction of iNOS since the phytocannabinoid (i) was pharmacologically active when given both 30 min before LPS as well as 15 h after the proinflammatory insult, that is once the enzyme had been already expressed and (ii) did not affect iNOS mRNA and protein expression, as revealed by RT-PCR and Western blot analyses. Likewise, CBC did not affect the expression of COX-2, another key enzyme involved in macrophage function. The latter result might be explained by the failure of CBC at reducing the LPS-induced IL-1β levels, since IL-1β represents one of the main proinflammatory cytokines able to induce COX-2 expression in macrophages (Samad *et al.*, 2001; Liu *et al.*, 2003). On the other hand, CBC reduced the levels of both IL-10 and INF-γ, two cytokines which limit or sustain, respectively, the inflammatory response in LPS-treated macrophages (Hawiger, 2001; Moore *et al.*, 2001). The ability of macrophages to overproduce IL-10 (an anti-inflammatory cytokine) in response to LPS has been previously documented (Brightbill *et al.*, 2000; Cao *et al.*, 2005; Aviello *et al.*, 2011) and can be considered as an adaptive reaction of the macrophages aiming at counteracting the inflammatory insult.

To investigate the mechanism of CBC-induced suppression of nitrite production in macrophages, we considered the possible involvement of cannabinoid (CB_1 and CB_2) and adenosine receptors as well as TRPA1 channels by evaluating the effect of selective receptor antagonists on CBC action as well as by exploring the expression of cannabinoid receptors and TRPA1 in LPS-treated macrophages.

Possible involvement of cannabinoid receptors in the CBC response in macrophages

CBC is a low (1 μM < K_i < 2 μM) and moderate (K_i~0.1 μM) affinity ligand for human CB_1 and CB_2 receptors respectively (V. Di Marzo, unpubl. data). Furthermore, CBC inhibits endocannabinoid reuptake, and thus might indirectly

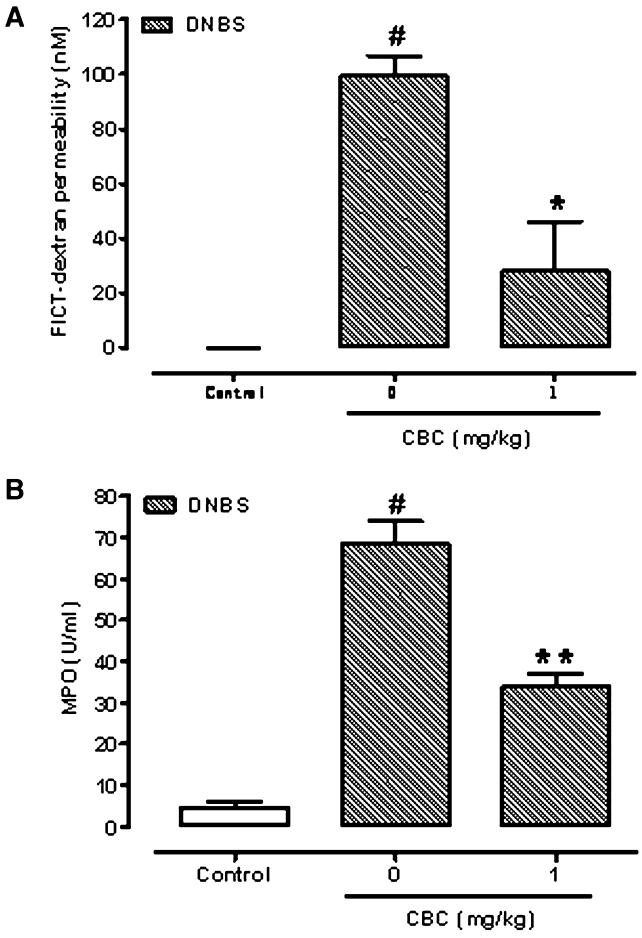

Figure 9

Inhibitory effect cannabichromene (CBC) on serum FICT-dextran concentration (a measure of intestinal barrier function) (A) and myeloperoxidase (MPO, a marker of intestinal inflammation) activity (B) in dinitrobenzene (DNBS)-induced colitis in mice. Permeability and MPO activity were measured on colonic tissues 3 days after vehicle or DNBS (150 mg·kg^{-1}, intracolonically). CBC (1 mg·kg^{-1}) was administered (i.p.) for 2 consecutive days starting 24 h after the inflammatory insult. Bars are mean ± SEM of five mice for each experimental group. $^{\#}P < 0.001$ versus control; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus DNBS alone.

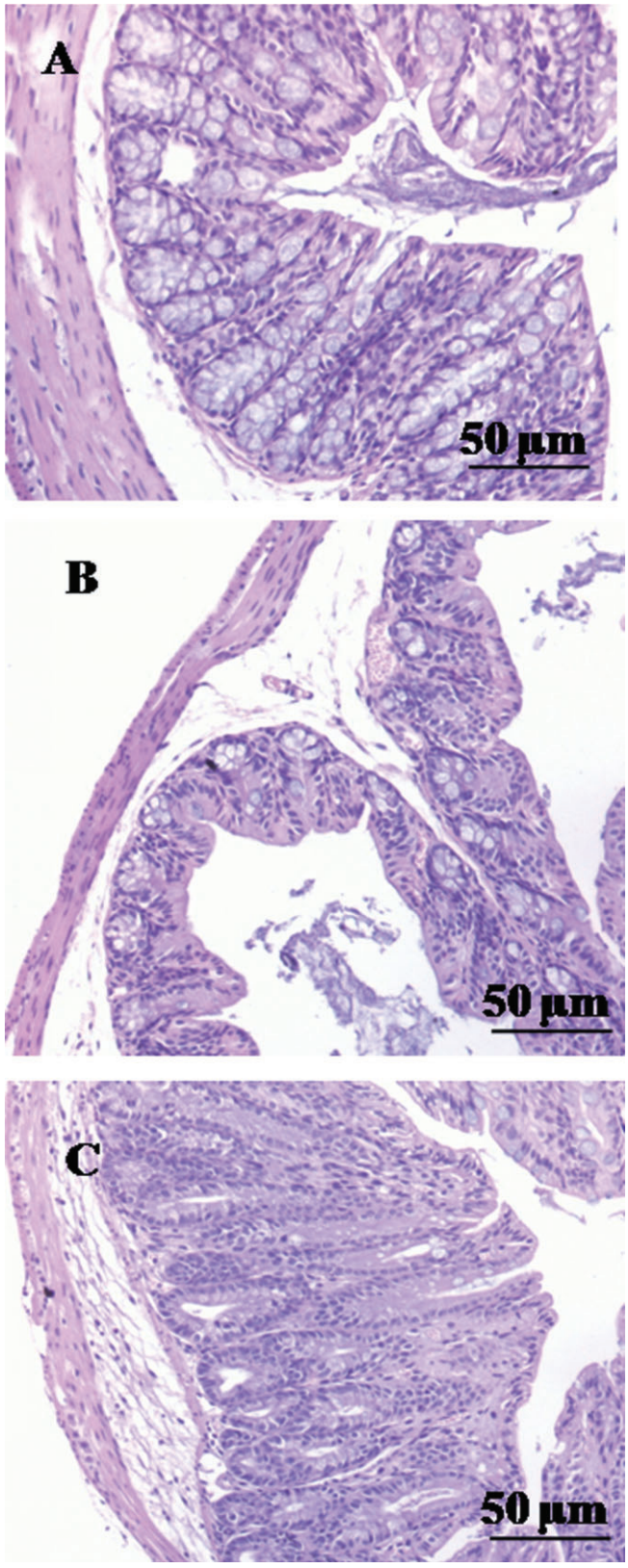

Figure 10

Histological evaluations of inflamed and non-inflamed colons: effect of cannabichromene (CBC). No histological modification was observed in the mucosa and submucosa of control mice (A); mucosal injury induced by dinitrobenzene sulfonic acid (DNBS) administration (B); treatment with CBC reduced colon injury stimulating a regeneration of the glands (C). CBC (1 mg·kg^{-1}) was administered (i.p.) for 2 consecutive days starting 24 h after the inflammatory insult. Histological analysis was performed 3 days after DNBS (150 mg·kg^{-1}, intracolonically). Original magnification ×200. The figure is representative of three experiments.

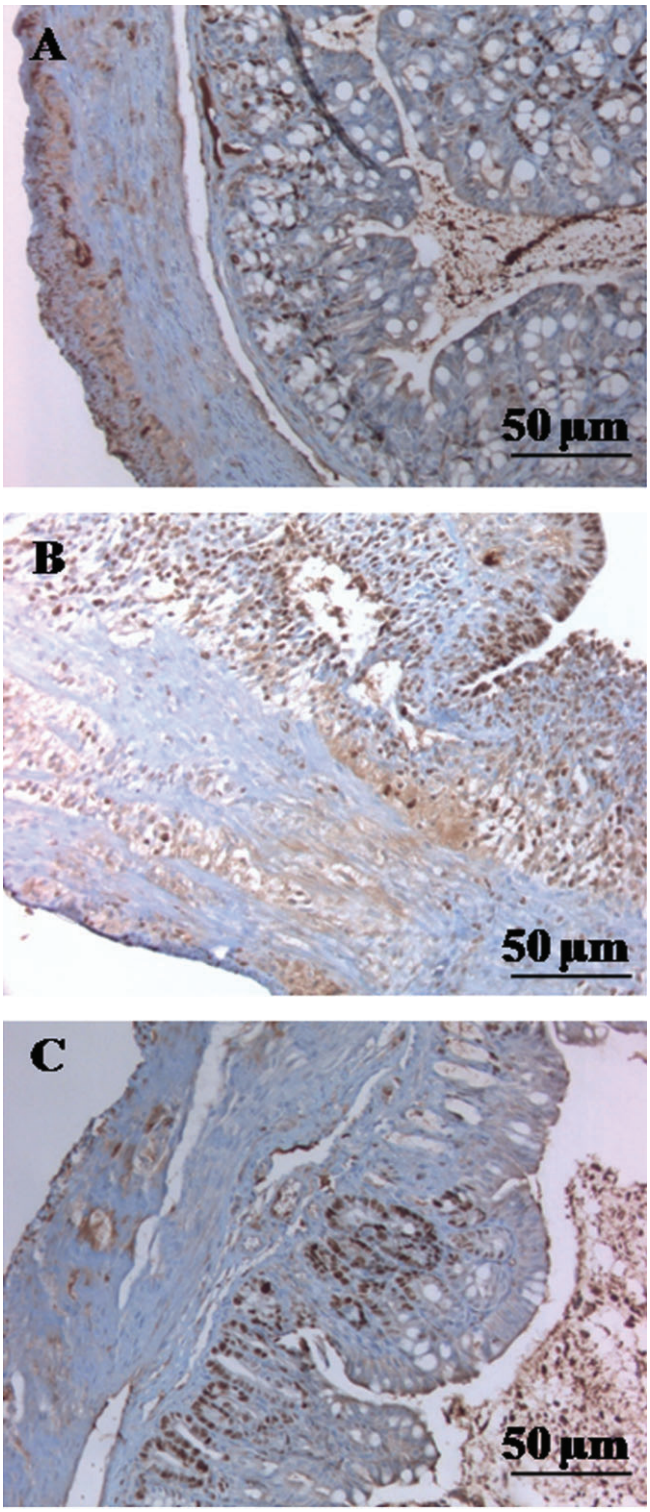

Figure 11

Different patterns of Ki-67 immunoreactivity in the colonic mucosa of control mice (A), dinitrobenzene sulfonic acid (DNBS)-treated mice (B) and mice treated with DNBS plus cannabichromene (C). (A) Ki-67 immunopositive cells localized to the lower of the crypts. (B) Ki-67 immunoreactivity was observed on inflammatory cells. (C) Ki-67 immunopositive cells observed only in the expanded basal zone. CBC (1 mg·kg^{-1}) was administered (i.p.) for 2 consecutive days starting 24 h after the inflammatory insult. The figure is representative of three experiments.

activate – via increased extracellular endocannabinoid levels – the cannabinoid receptors (Ligresti *et al.*, 2006; De Petrocellis *et al.*, 2011). CBC was shown to stimulate descending pathways of antinociception and to cause analgaesia in rats in a manner partly attenuated by a CB_1 receptor antagonist (Maione *et al.*, 2011). We have shown here that the inhibitory effect of CBC was mimicked by selective CB_1 and CB_2 receptor agonists, suggesting that a direct pharmacological activation of such receptors results in inhibition of nitrite production. The ability of direct activation of both CB_1 and CB_2 receptor to reduce nitrite production in activated macrophages was previously documented (Ross *et al.*, 2000; Aviello *et al.*, 2011). Surprisingly, however, we observed that the inhibitory effect of CBC was further increased by rimonabant and AM251 (two CB_1 receptor antagonists), at concentrations that, however, were inactive *per se*. These results, while confirming that exogenous activation of CB_1 reduces NO formation in macrophages, negate the possibility that CBC acts via direct or indirect activation of CB_1 receptors. This hypothesis is also supported by the results we obtained in the [^{35}S]GTPγS binding assay performed with hCB_1-CHO cell membranes. Thus, we found that CBC, at concentrations that included the one at which it significantly inhibits nitric oxide production (1 µM), did not induce any significant activation of cannabinoid CB_1 receptors in this assay. Moreover, using the same assay, we also found that when CBC was administered 30 min after 1 µM AM251 or 0.1 µM rimonabant, it did not significantly affect the E_{max} of either of these compounds for their inhibition of [^{35}S]GTPγS binding. It might be possible that an endogenous CB_1 tone exists, which may couple negatively to the CBC signalling pathway and counteract CBC inhibition of nitrite production. Indeed, we found that LPS enhances anandamide levels in macrophages, and that CBC, instead, only elevates OEA levels. According to some authors, also OEA, but not PEA (the levels of which were not elevated by CBC) is taken up by cells through the same mechanism responsible for anandamide uptake (Hillard *et al.*, 1997; Alhouayek and Muccioli, 2012). It is possible that CBC could not elevate anandamide levels because these were already maximally up-regulated by LPS. OEA, which is chemically related to anandamide, was previously shown to produce anti-inflammatory effects (Lo Verme *et al.*, 2005) and hence, it is possible that a part of the beneficial effect of CBC observed here in macrophages could be due to its ability to increase OEA levels. It is also possible that CBC can merely synergize with rimonabant by unmasking the anti-inflammatory action of a *per se* inactive dose of this antagonist. In agreement with this hypothesis, rimonabant, but not the CB_2 receptor antagonist SR144528, was previously shown to inhibit LPS-induced inflammation in wild-type mice, but not CB_1 null mice (Croci *et al.*, 2003). Accordingly, we also found here that SR144528 did not change the inhibitory effect of CBC on nitrite production in LPS-challenged macrophages. In another model of LPS-induced inflammation (LPS-induced paw oedema), DeLong *et al.* (2010) have

recently shown that the anti-inflammatory action of CBC *in vivo* (LPS-induced paw oedema) was not blocked by either SR144528 or rimonabant.

In order to give further insights into the role of cannabinoid receptors in CBC action, we evaluated the effect of this plant constituent on cannabinoid receptors mRNA expression in macrophages. It was recently demonstrated that CBC alters the mRNA expression of cannabinoid receptors in the inflamed gut (Izzo *et al.*, 2012). In the present study, we have shown that LPS causes up-regulation of CB_1 receptors and down-regulation of CB_2 receptors and that those changes were not modified by CBC. These results rule against the possibility that CBC could exert anti-inflammatory actions in macrophages by altering cannabinoid mRNA receptor expression.

Possible involvement of TRPA1 in the CBC response in macrophages

CBC was shown to potently activate TRPA1 channels, as revealed by the increase in $[Ca^{2+}]$ in human embryonic kidney (HEK)-293 cells overexpressing recombinant rat TRPA1 (De Petrocellis *et al.*, 2008; De Petrocellis *et al.*, 2011). CBC was also found to exert antinociceptive effects in the tail flick test via TRPA1 (Maione *et al.*, 2011). Allyl isothiocyanate, a TRPA1 agonist, was previously shown to reduce nitrite production in LPS-activated macrophages (Ippoushi *et al.*, 2002). In the present study, we have shown that the effect of CBC on nitrite production was mimicked by other TRPA1 agonists, namely carvacrol and cinnamaldehyde (Moran *et al.*, 2011). However, also AP-18 (10 and 20 μM) and HC-030031 (10 and 20 μM), two well-established TRPA1 antagonists (Alexander *et al.*, 2011; Moran *et al.*, 2011), completely blocked nitrite production at a concentration (10 μM) below the IC_{50} value required to antagonize the agonists HEK-293 cells overexpressing TRPA1 channels (16.5 μM for AP-18 and 13.3 μM for HC-030031) (Capasso *et al.*, 2012). The capability of the two TRPA1 antagonists to efficiently block nitrite production at very low concentrations precluded us the possibility to evaluate the effect of CBC in the presence of AP-18 or HC-030031. It should be also emphasized that the ability of TRPA1 agonists and antagonists to have a pharmacological effect in the same direction is not surprising since it is well known that TRP agonists (via a desensitizing mechanism) and antagonists (via receptor blockade) can interfere with TRP channel function (Moran *et al.*, 2011). Accordingly, CBC was shown to desensitize TRPA1 receptors in HEK-293 cells overexpressing TRPA1 channels after an incubation as short as 5 min (De Petrocellis *et al.*, 2012).

We also investigated TRPA1 mRNA expression in macrophages. Previous *in vivo* studies found that CBC may change TRPA1 as well as other TRP channel (i.e. transient receptor potential cation channel, subfamily V, member 3 and member 4) mRNA expression in the inflamed gut (De Petrocellis *et al.*, 2012; Izzo *et al.*, 2012), thus providing another potential mechanism – in addition to direct activation – through which this phytocannabinoid can exert pharmacological actions. In the present study, we have observed a very faint expression of TRPA1 mRNA expression in macrophages which was abolished by LPS and not restored by CBC treatment. It is possible that the further reduction of TRPA1 mRNA levels did not result in a complete removal also of TRPA1 protein (i.e. during *in vitro* culture of the cells the transcription of TRPA1 mRNA could be depressed while residual TRPA1 protein could be still present, a possibility that we could not investigate due to the lack of a commercially available specific and reliable antibody against the mouse TRPA1), thus explaining why the TRPA1 agonists and antagonists tested here did reduce LPS-induced inflammation.

In summary, we have shown that both TRPA1 antagonists and agonists, including CBC, inhibit nitrite productions in macrophages. Whether or not TRPA1 channels are involved in such action has not been conclusively demonstrated in the present study. While the similarity of the effect of TRPA1 agonists and antagonists could be explained by the ability of agonists, including CBC, to activate and subsequently desensitize TRPA1 channels, the undetectable TRPA1 mRNA signal in macrophages activated by LPS seems to rule against this possibility.

Possible involvement of adenosine receptors in the CBC response in macrophages

We also investigated the possible involvement of adenosine receptors. CBC was found to exerts analgesic actions in a CGS 15943 (adenosine receptors antagonist)-sensitive way (Maione *et al.*, 2011). However, we found that the CGS 15943 did not modify the inhibitory effect of CBC on nitrite production, thus excluding a role of these receptors in CBC anti-inflammatory effects.

CBC ameliorates experimental murine colitis

We investigated the effect of CBC in an experimental model of IBD for a number of reasons. First, macrophage targeting treatment ameliorates colonic inflammation in experimental colitis models and the regulation of abnormal responses of macrophages appears to be a promising therapeutic approach for the treatment of IBD (Yoshino *et al.*, 2010). Second, *Cannabis* is commonly used by IBD patients for symptom relief (Lal *et al.*, 2011; Naftali *et al.*, 2011) and *Cannabis* inhalation improves clinical disease activity and quality of life in patients with long-standing IBD (Lahat *et al.*, 2012). Third, both endocannabinoids and TRPA1 (i.e. the main pharmacological targets of CBC) are involved in experimental colitis (D'Argenio *et al.*, 2006; Di Marzo and Izzo, 2006; Izzo and Camilleri, 2009; Engel *et al.*, 2011; Holzer, 2011). Fourth, we recently demonstrated that CBC inhibited gastrointestinal transit in an experimental model of intestinal inflammation (Izzo *et al.*, 2012). Other *Cannabis* constituents, namely Δ^9-THC and CBD, were previously shown to ameliorate experimental colitis in rodents after i.p. administration (Borrelli *et al.*, 2009; Jamontt *et al.*, 2010; Schicho and Storr, 2012), a route of administration able to bypass the hepatic metabolism, a limiting factor for oral cannabinoid use (Huestis, 2005). Interestingly, it has been recently reported that intrarectal or intraperitoneal (but not oral) administration of CBD, another plant-derived cannabinoid, improves murine colitis induced by trinitrobenzene sulfonic acid (Schicho and Storr, 2012). We have found here that intraperitoneal CBC exerts a therapeutic effect in the DNBS model of colitis as revealed by the reduction of colon weight/colon length *ratio* (a simple and reliable parameter of inflammation), intestinal permeability (a measure of intestinal epithe-

lial integrity) (Osanai *et al.*, 2007) and MPO, an index of neutrophil infiltration (Krawisz *et al.*, 1984) as well as by histology and immunohistochemistry. Importantly, histological and immunohistochemical analyses showed the ability of CBC to induce tissue regeneration in the inflamed gut.

CBC significantly cured experimental colitis at the 1 mg·kg^{-1}daily dose, which is a dose more than 100-fold lower than the subacute LD_{50} dose calculated in mice receiving, for 7 days, repeated intraperitoneal daily dosing of CBC (Krawisz *et al.*, 1984). In other *in vivo* assays (i.e. paw oedema and intestinal motility in the inflamed gut), CBC was shown to exert statistically significant pharmacological actions starting from the 10 mg·kg^{-1}dose (DeLong *et al.*, 2010; Izzo *et al.*, 2012).

Conclusions

In the present study, we have shown that the non-psychotropic *Cannabis* constituent CBC reduced nitric oxide, IL-10 and interferon-γ levels in peritoneal macrophages activated by LPS. The effect of CBC on nitric oxide production is mimicked by other TRPA1 ligands and does not appear to be mediated by direct or indirect cannabinoid receptor activation, although it is apparently modulated by CB_1 receptors. Specifically, since the CBC response was exacerbated in the presence of CB_1 antagonists, it is possible that an endogenous cannabinoid 'tone' at CB_1 is coupled negatively to CBC pharmacological actions. *In vivo*, CBC exerted protective effects in experimental colitis at a dose more than 100-fold lower than the LD_{50} value previously reported. At least two significant therapeutic implications are evidenced by the present study: first, by decreasing NO production, CBC might limit tissue destruction caused by NO in autoimmune diseases; and second, in the light of its curative effect on murine colitis *in vivo*, CBC can be regarded as a promising candidate for clinical evaluation in IBD patients.

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Conflict of interest

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