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ORIGINAL ARTICLE

Local administration of WIN 55,212-2 reduces chronic granuloma-associated angiogenesis in rat by inhibiting NF-κB activation

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Abstract Chronic inflammation is often associated with granuloma formation that is a hallmark of many human diseases. The transcription factor nuclear factor-kappa B (NF-κB) plays a central role in this process by regulating the expression of several pro-inflammatory genes. Cannabinoids (CBs) from *Cannabis sativa* L. exert a large number of biological effects including anti-inflammatory and anti-angiogenic effects. In this study, we investigated the role of CBs on granuloma formation induced by λ-carrageenin-soaked sponge implant in rat. Our results show that local administration of WIN 55,212-2, a CB_1/CB_2 agonist, given daily or at time of implantation significantly decreased weight and neo-angiogenesis in granuloma tissue and inhibited nuclear factor-kappa B (NF-κB)/DNA binding that was associated with a reduced inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), tumor

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necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF) messenger RNA (mRNA) and protein expression. Also, arachidonyl-2-chloroethylamide (ACEA), a CB_1 selective agonist, and JWH-015, a CB_2 selective agonist, exhibited the same effects that were reversed by SR141716-A and SR144528, respectively, CB_1 and CB_2 selective antagonists. These results indicate that CBs given locally may represent a potential therapeutic tool in controlling chronic inflammation avoiding psychotropic effects.

Keywords Cannabinoids · Chronic inflammation · Angiogenesis · Nuclear factor-kappa B

Introduction

Chronic inflammation, due to a variety of etiological agents, represents one of the most diffuse pathology. A specific hallmark of the inflammatory process is represented by granulomatous tissue formation that is well-characterised histologically by the presence of infiltrating macrophages, epithelial and giant cells, and activated mast cells (MC) surrounded by a lymphocyte mantle and proliferating fibroblasts [1]. Angiogenesis is observed during granulomatous inflammation. It allows the maintenance of tissue perfusion and cellular traffic supporting chronicity [2–4]. Chronic granuloma formation and angiogenesis are characterized by the prolonged release of a wide range of pro-inflammatory and pro-oxidant mediators [5] from phagocytic cells [6], endothelial cells [7], and mast cells [8], concomitant with a diminished production of anti-inflammatory and antioxidant mediators [9]. It has been shown that the transcription factor NF-κB plays a central role in most physio-pathological processes, including granuloma formation [10]. NF-κB up regulates the expression of several genes involved in chronic inflammation and angiogenesis, including tumor necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF), adhesion molecules, chemokines, growth factors, and inducible enzymes as cyclooxygenase 2 (COX-2) and nitric oxide syntase-2 (iNOS) [11–13]. These inflammatory mediators are, in turn, able to regulate NF-κB activation [14]. Cannabinoids (CBs), the active components of *Cannabis sativa* L. (marijuana) and their derivatives, exert a wide array of effects on the central nervous system (CNS) as well as on peripheral sites such as the immune, cardiovascular, digestive, reproductive, and ocular systems [15]. Most CB effects are mediated by the activation of specific G-protein-coupled receptors that normally bind to a family of endogenous ligands: the endocannabinoids [16]. Two types of CB receptors have been identified: the CB_1 receptor, mostly expressed in the brain and responsible for CB psychoactivity [17], and the CB_2 receptor, mostly present in the immune system and not involved in CB psychoactivity [18]. However, the expression of CB_1 and CB_2 receptors on the cells from the immune system has been extensively documented [19]. Previous studies demonstrated that CBs, such as Δ^9THC and a synthetic analogue, CP55,940 [20], modulate immune response in vivo and in vitro. In fact, they inhibit proliferative responses of T lymphocytes [21], cytotoxic T cell activity [22], macrophage function, antigen presentation [23], and NO production by macrophages [24]. Furthermore, CBs have been proposed as anti-inflammatory agents in several models of acute inflammation, as carrageenin edema [25], and chronic immune inflammation, as arthritis [26]. CBs also inhibit tumor angiogenesis in vivo by two mechanisms: a direct inhibition of vascular endothelial cell migration and suppression of the release of pro-angiogenic factors, VEGF and MMP [27]. In the light of these observations, we studied the effects of some CB agonists in λ-carrageenin-induced granuloma, a model of non-immune chronic inflammation. Specifically, WIN 55,212-2, a non-selective synthetic CB_1/CB_2 agonist, arachidonyl-2-chloroethylamide (ACEA) and JWH-015, CB_1 and CB_2 selective agonists, and SR141716-A and SR144528 selective antagonists were used.

Materials and methods

Sponge implantation

Male Wistar rats (Harlan, Italy) weighing 200–220 g were used in all experiments. The animals were provided with food and water ad libitum. The light cycle was automatically controlled (on, 0700 hours; off, 1900 hours), and the room temperature was thermostatically regulated to 22±1°C with 60±5% humidity. Before the experiments, the animals were housed in these conditions for 3–4 days to become acclimatized. Sponges were implanted as previously described by Russo et al. [8]. Briefly, two polyether sponges (0.5×1.5×2.0 cm) weighing 0.035±0.002 g were implanted subcutaneously on the back of rats (n=12–18 for each group) under general anesthesia. Sponges and surgery tools were sterilized by autoclaving for 20 min at 120°C. λ-Carrageenin (1% w/v; Sigma) was dissolved in pyrogen-free saline (0.5 ml/sponge), in the presence or absence of CB agonists and/or antagonists, and injected into each sponge; saline (0.5 ml/sponge) was used as control. Ninety-six hours after sponge implant, the rats were sacrificed in an atmosphere of CO_2. The granulomatous tissue around the sponge was dissected by using a surgical blade, weighted, quickly frozen in liquid nitrogen, and stored at −80°C. Animal care, as well as all experiments, was in accordance with the European Community Council

directive 86/609/EEC, and efforts were made to minimize animal suffering and to reduce the number of animals used.

Treatments

The non-selective CB agonist WIN 55,212-2 (CB_1/CB_2; Tocris) was given intraperitoneally daily (5 mg/kg) or locally (0.01–0.1–1 mg/ml), daily at time 0, i.e., only once after sponge implantation, or, in other experiments, 3 and 4 days after λ-carrageenin 1% administration, in a volume of 100 μl/sponge. For each experiment, we used three animals/treatment: ACEA (0.1 mg/ml), a CB_1 full agonist, and JWH-015 (0.1 mg/ml), a CB_2 full agonist, were given only once at time 0. SR141716A (0.1 mg/ml) and SR144528 (0.1 mg/ml), a CB_1 and a CB_2 full antagonist, respectively, were co-somministrated with WIN 55,212-2 (0.1 mg/ml) only once at time 0. WIN 55,212-3, the inactive enantiomer of WIN 55,212-2, was used as control. All CB agonists (Tocris) and antagonists (Sanofi) were dissolved in ethanol. The highest concentration of ethanol in the sponges was 10%; therefore, the vehicle alone was administered to animals to verify if it may interfere with granuloma formation.

Evaluation of angiogenesis

Angiogenesis was evaluated by both histological investigations and hemoglobin content measurement. The granulomatous tissue, i.e., the new formation tissue encapsulating the sponge, was collected and measured with a balance weighing a minimum of 0.02 to a maximum of 300 g (KERN EG300-EM), always by the same person who was blinded for the treatments. In some experiments, the granulomatous tissue was fixed in formol–methanol (9:1, v/v) solution at 4°C for 24 h. After dehydration in an ethanol series and infiltration with xylol, paraffin wax sections were cut at 4–6 μm and stained with hematoxylin and eosin. Vessels counting was performed on five randomly selected sections using a 100× objective lens.

In some experiment, the granulomatous tissue was homogenized on ice with the Polytron PT300 tissue homogenizer in 1× PBS (4 ml each gram of wet weight)

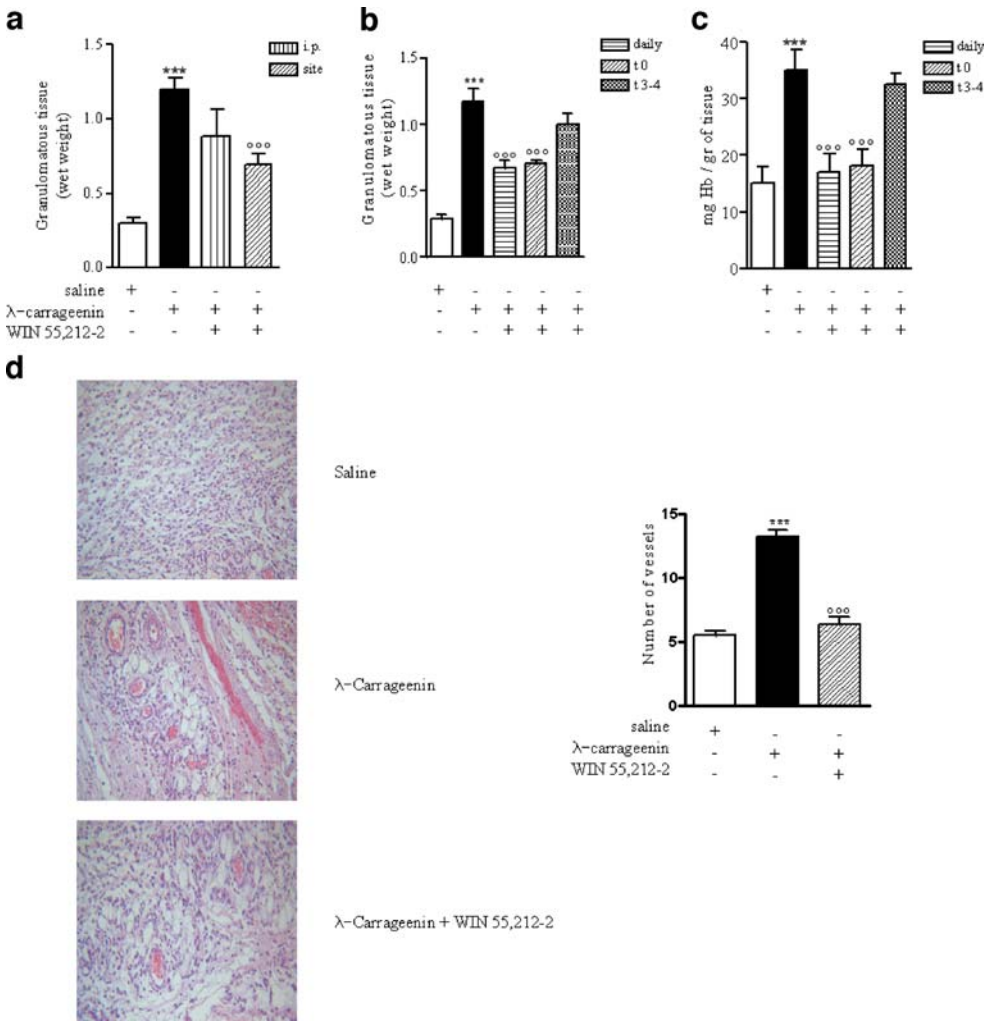

Fig. 1 Effects of WIN 55,212-2 on λ-carrageenin-induced granulomatous tissue formation. **a** WIN 55,212-2 was administrated daily intra-peritoneally (i.p.; 5 mg/kg) or locally (site; 0.1 mg/ml). **b** and **c** WIN 55,212-2 (0.1 mg/ml) was injected locally in daily administration (daily), single administration at time of implantation (t=0), or single administration 3 and 4 days after implantation (*t*=3–4). Granulomatous tissue formation was evaluated 96 h after implantation as wet weight of tissue around the sponge in **a** and **b** and as hemoglobin content in **c**. Data are expressed as mean±SEM of $n=3$ separate experiments; ***$p<0.001$ vs saline, °°°$p<0.001$ vs λ-carrageenin alone. **d** A representative histological analysis and relative counting of vessels in granulomatous tissue from saline-, λ-carrageenin-, and λ-carrageenin+WIN-55,212-2-treated sponges at *t*=0. Fields are representative of three separate experiments. Original magnification, 100×

as previously described [28]. Briefly, after centrifugation at 2,500×*g* for 20 min at 4°C, the supernatant was further centrifuged at 5,000×*g* for 30 min, and hemoglobin concentration in the supernatant was determined spectrophotometrically at 450 nm by using the hemoglobin assay kit (Sigma Diagnostic). The hemoglobin content was expressed as milligram hemoglobin per gram of wet weight.

Myeloperoxidase activity

Myeloperoxidase (MPO), a marker of polymorphonuclear (PMN) leukocyte accumulation, was determined as previously described by Mullane et al. [29]. Briefly, 96 h after sponge implantation, the granulomatous tissue was removed and weighed. The tissue was homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000×*g* at 37°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was determined as the amount of enzyme degrading 1 mmol of peroxide per min at 37°C and was expressed in milliunits per 100 mg of wet tissue weight.

Preparation of cytosolic and nuclear extracts

For homogenization, tissue was suspended in ice-cold hypotonic lysis buffer [20 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), 100 mM $MgCl_2$, 0.4 M NaCl, 0.5 mM phenylmethylsulphonylfluoride, 15 μg/ml soybean trypsin inhibitor, 3 μg/ml pepstatin A, 2 μg/ml leupeptin, 40 μM benzamidine, 1 mM dithiothreitol, 1% Nonidet P40, 20% glicerol) in a ratio of 0.4 ml per 100 μg of tissue and homogenized at the highest setting for 2–5 min in Polytron PT300 tissue homogenizer. Protein concentration was determined using the BioRad protein assay kit. After homogenization the cytoplasmic fraction was then obtained by centrifugation for 1 min at 13,000×*g*. The nuclear pellet was resuspended in 60 μl of high salt extraction buffer [20 mM HEPES at pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 25% v/v glycerol, 0.5 mM phenylmethylsulphonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol (DTT)] and incubated with shaking at 4°C for 30 min. The nuclear extract was then centrifuged for 15 min at 13,000×*g*, and the supernatant was aliquoted and stored at −80°C. The protein concentration was determined by the BioRad protein assay kit.

Electrophoretic mobility shift assay

Double-stranded oligonucleotide containing the NF-κB recognition sequence (5′-CAACGGCAGGGGAACTCCC TCTCCTT-3′) was end-labeled with ^{32}P-γ-ATP. Nuclear extracts (5 μg) were incubated for 15 min with radiolabeled oligonucleotides (2.5–5.0×10^4 cpm) in 20 ml reaction buffer containing 2 mg poly dI–dC, 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DL–dithiothreitol, 1 mg/ml bovine serum albumin, and 10% (v/v) glycerol. The specificity of the NF-κB/DNA binding was determined by competition reaction in which a 50-fold molar excess of unlabeled wild-type, mutant, or Sp-1 oligonucleotide was added to the binding reaction 15 min before the radiolabeled probe. In supershift assay, antibodies reactive to p50 or p65 proteins were added to the reaction mixture 15 min before the

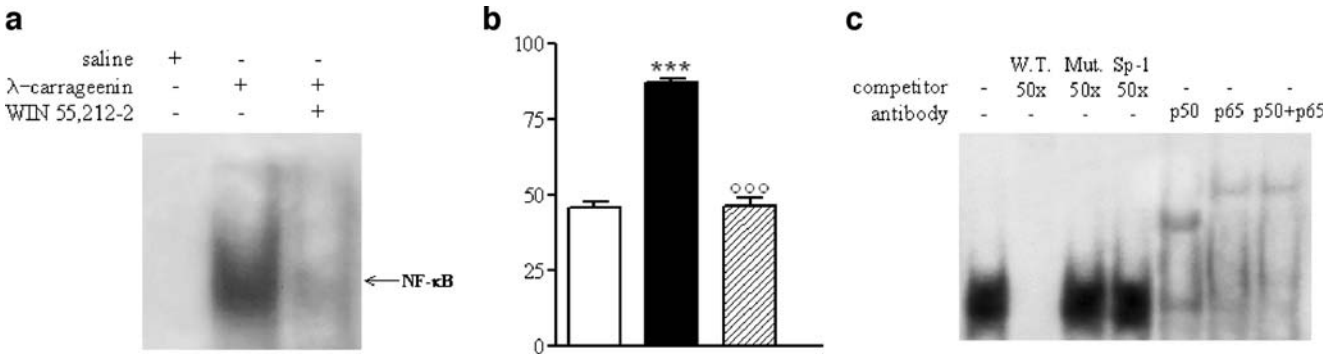

Fig. 2 Effects of WIN 55,212-2 on λ-carrageenin-induced NF-κB activation in granuloma tissue. The representative electrophoretic mobility shift assay (**a**) and the densitometric analysis of the corresponding bands (optical density; **b**) showing NF-κB/DNA-binding activity in nuclear extracts from granulomatous tissue at 96 h. Data in **a** are representative of three separate experiments. The results in **b** are expressed as mean±SEM of n=3 separate experiments; ***p<0.001 vs saline and °°°p<0.001 vs λ-carrageenin alone. **c**) Characterization of NF-κB/DNA complex was performed in tissue from λ-carrageenin-soaked sponges. In competition reaction, nuclear extracts were incubated with radiolabeled NF-κB probe in the absence or presence of identical but unlabeled oligonucleotide (W.T., 50×), mutated non-functional NF-κB probe (Mut., 50×), or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50×). In supershift experiments, nuclear extracts were incubated with antibodies against p50, p65, and p50+p65 15 min before incubation with radiolabeled NF-κB probe. Data are from a single experiment and are representative of three separate experiments

addition of the radiolabeled NF-κB probe. Nuclear protein–oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 1× Tris–borate–EDTA buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with an intensifying screen at −80°C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with GS-700 imaging densitometer (BioRad) and a computer programme (Molecular Analyst, IBM).

Western blot analysis

The immunoblotting analysis of COX-2, iNOS, TNF-α, VEGF, p50, p65, histone-1, and β-actin proteins was performed on total protein fractions of granulomatous tissue homogenates. Total proteins were mixed with gel loading buffer (50 mM Tris/10% SDS/10% glycerol 2-mercaptoethanol/2 mg bromophenol for ml) in a ratio of 1:1 and boiled for 3 min. Equivalent amounts (50 μg) of each sample were electrophoresed through a 8–12% discontinuous polyacrilamide minigel. Proteins were transferred onto nitrocellulose membrane, according to the manufacturer's instructions (BioRad). The membranes were saturated by incubation at 4°C overnight with 10% non-fat dry milk in 1× PBS and then incubated with the appropriate antiserum: anti-mouse VEGF antiserum (1:200 v/v, Neo-Marker), anti-mouse TNF-α (1:250 v/v, NeoMarker), anti-mouse iNOS (1:2,000 v/v, BD Bioscence), anti-rabbit COX-2 (1:250 v/v, BD Bioscence), anti-mouse p50 and anti-mouse p65 (1:1,000 v/v, Santa Cruz), anti-rabbit histone-1 (1:1,000 v/v, Santa Cruz), and anti-mouse β-actin (1:1,000 v/v, Santa Cruz) for 2 h at room temperature. The membranes were washed three times with 1% Triton X-100 in 1× PBS and then incubated with anti-mouse or anti-rabbit immunoglobulins coupled to peroxidase (Dako; 1:2,000 v/v). The immune complexes were revealed by using enhanced chemiluminescence detection reagents (Amersham) according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands on X-ray film were scanned and analyzed by densitometric analysis with a GS-700 imaging densitometer.

mRNA analysis

The iNOS, COX-2, TNF-α, and VEGF mRNA level in granulomatous tissue was determined by using the semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) method. Total RNA was extracted from tissue samples by the use of an ultrapure TRIzol reagent (GibcoBRL) as directed by the manufacturer. RNA (5 μg) was then reverse-transcribed in 20 μl with 200 U of superscript II RNase H-reverse transcriptase (Invitrogen) in the presence of random hexamers (5 μM), 20 U of RNasin (Promega), dNTPs (10 mM), for 1 h at 42°C. PCR was performed on 2 μl of the reverse transcription (RT) reaction mixture in a final volume of 50 μl with 2.5 U of *Taq* polymerase (Roche) and 5 μM of the appropriate primers as follows: iNOS, 5′-GTGTTCCACCAGGAGATGTTG-3′ (forward primer) and 5′-CTCCTGCCCACTGAGTT CGTC-3′ (reverse primer); COX-2, 5′-GCCACCTCTG CGATGCTCTT-3′ (forward primer) and 5′-GTGTT TGGGGTGGGCTTCAG-3′ (reverse primer); TNF-α, 5′-CAAGGAGGAGAAGTTCCCAA-3′ (forward primer) and 5′-CGGACTCCGTGATGTCTAAG-3′ (reverse primer); VEGF, 5′-CGCGAATTCCATGAACTTTCTGCTCTCT-3′ (forward primer) and 5′-TGAGAATTCTAGTTCCCGA AACCCTGA-3′ (reverse primer); and β-actin, 5′-GGCAC CACACCTTCTACA-3′ (forward primer) and 5′-CAGG AGGAGCAATGATCT-3′ (reverse primer). To obtain linear amplification curves, the complementary DNA (cDNA) mixtures were subjected to 10, 15, and 20 cycles for the control β-actin and 20, 25, and 30 cycles for all other genes under the

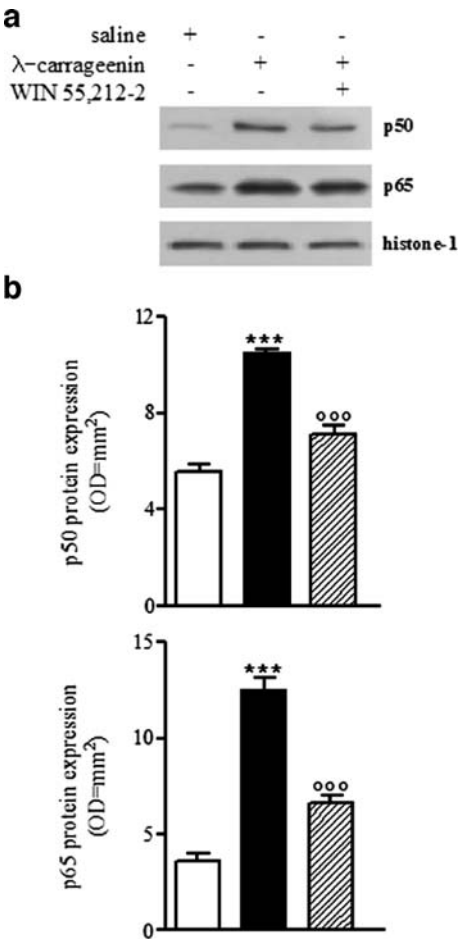

Fig. 3 Effects of WIN 55,212-2 on λ-carrageenin-induced NF-κB subunits nuclear translocation. The representative Western blot (**a**) and relative densitometric analysis (**b**) show p50 and p65 nuclear level in granulomatous tissue at 96 h. Histone-1 expression is shown as control. Data in **a** are from a single experiment and are representative of three separate experiments. The results in **b** are expressed as mean± SEM of three separate experiments; ***$p<0.001$ vs saline, °°°$p<0.001$ vs λ-carrageenin alone

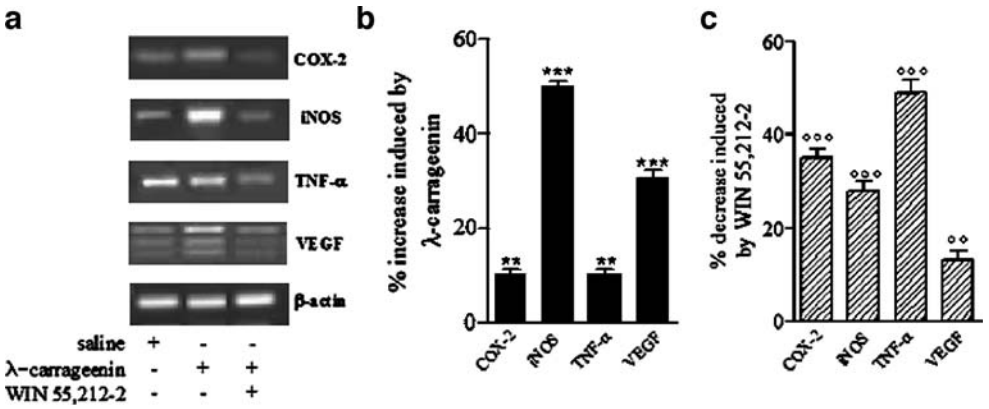

Fig. 4 Effects of WIN 55,212-2 on λ-carrageenin-induced COX-2, iNOS, TNF-α, and VEGF mRNA expression in granulomatous tissue at 96 h. **a** Representative Vistra green-stained agarose gel of RT-PCR products corresponding to COX-2, iNOS, TNF-α, and VEGF mRNA in granulomatous tissue from sponges injected with saline, λ-carrageenin (1%), λ-carrageenin+WIN 55,212-2 (0.1 mg/ml). The expression of β-actin was used as a control for cDNA amount. PCR was allowed to proceed for 10, 15, and 20 cycles using β-actin primers and for 25, 30, and 35 cycles using specific primers for all other genes. Shown are signals corresponding to amplification cycles within the linear range (Materials and methods). **b** Quantitative analysis of the results of three independent experiments expressed in percent after normalization to β-actin mRNA levels calibrated to the signal of animals injected with saline solution (0%). **c** Quantitative analysis of the results of three independent experiments expressed in percent after normalization to β-actin mRNA levels calibrated to the signal of animals injected with λ-carrageenin. $^{**}p<0.01$, $^{***}p<0.001$ vs saline; $^{\circ\circ}p<0.01$, $^{\circ\circ\circ}p<0.001$ vs λ-carrageenin alone

following conditions: denaturating at 95°C for 1 min, annealing at 52 or 56°C for 1 min for COX-2 and iNOS or TNFα and VEGF, respectively, and extension at 72°C for 1 min. The final extension step was performed at 72°C for 10 min. Fifteen microliters of aliquots of PCR products were electrophoretically fractionated through 1% agarose gel containing the fluorescent Vistra green dye (Amersham Pharmacia Biotech) [30]. The labeling intensity of the PCR product, which is linear to the amount of DNA, was quantified using the Molecular Imager FX and Quantity One software (BioRad).

Statistical

The results were expressed as the mean±SEM of *n* animals, where each value is the average of responses in duplicate sites. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. $P<0.05$ was considered to be significant.

Results

Effects of systemic and local administration of WIN 55,212-2 on λ-carrageenin-induced granuloma formation

The implant of λ-carrageenin (1%)-soaked sponges on the back of rats caused, at 96 h, a significant increase of granuloma formation, evaluated as the wet weight of tissue around the sponge and compared to implants of saline-soaked sponges (1.39±0.14 g vs 0.38±0.03 g; $n=24$–36 sponges from 12–18 rats). The local daily administration of WIN 55,212-2 (0.1 mg/ml), a non-selective CB_1/CB_2 agonist, resulted in a significant reduction of granuloma formation (by 57.5%; $p<0.001$; $n=24$ sponges from 12 rats), while the intraperitoneal administration resulted ineffective (5 mg/ml; Fig. 1a). WIN 55,212-3 (0.1 mg/ml), the inactive enantiomer of WIN 55,212-2, did not reduce granuloma formation (data not shown) when given at time 0. In subsequent experiments, CB agonists were given locally. The ethanol vehicle did not affect the granuloma formation.

Time-dependent effects of locally injected WIN 55,212-2 on λ-carrageenin-induced granuloma formation

Figure 1b shows the results of the time-course experiments performed to analyze the effects of WIN 55,212-2 given locally according to the following schedule: (1) daily administration, (2) single administration at the time of implantation ($t=0$), or (3) administration 3 and 4 days after implantation ($t=3$–4). WIN 55,212-2 (0.1 mg/ml) strongly reduced granuloma formation when given daily (42.5% inhibition; $p<0.001$; $n=24$ sponges from 12 rats) or at time 0 (41.7 % inhibition; $p<0.001$; $n=24$ sponges from 12 rats), whereas it was ineffective when administered 3 and 4 days after sponge implantation. Moreover, WIN 55,212-2 (0.01, 0.1, 1 mg/ml) given locally and at time 0 caused a dose–related inhibition of granuloma formation (0.95± 0.09 g, 0.77±0.02 g, 0.39±0.04 g; $n=12$–16 sponges from 6–8 rats), compared to λ-carrageenin-soaked sponges. New micro-vessel formation was valuated by hemoglobin content and histological analysis. WIN 55,212-2 (0.1 mg/ml) strongly reduced hemoglobin content in granulomatous tissue when given daily (55.6% inhibition, $p<0.001$, $n=24$ sponges from 12 rats) or at time 0 (47.9% inhibition, $p<$

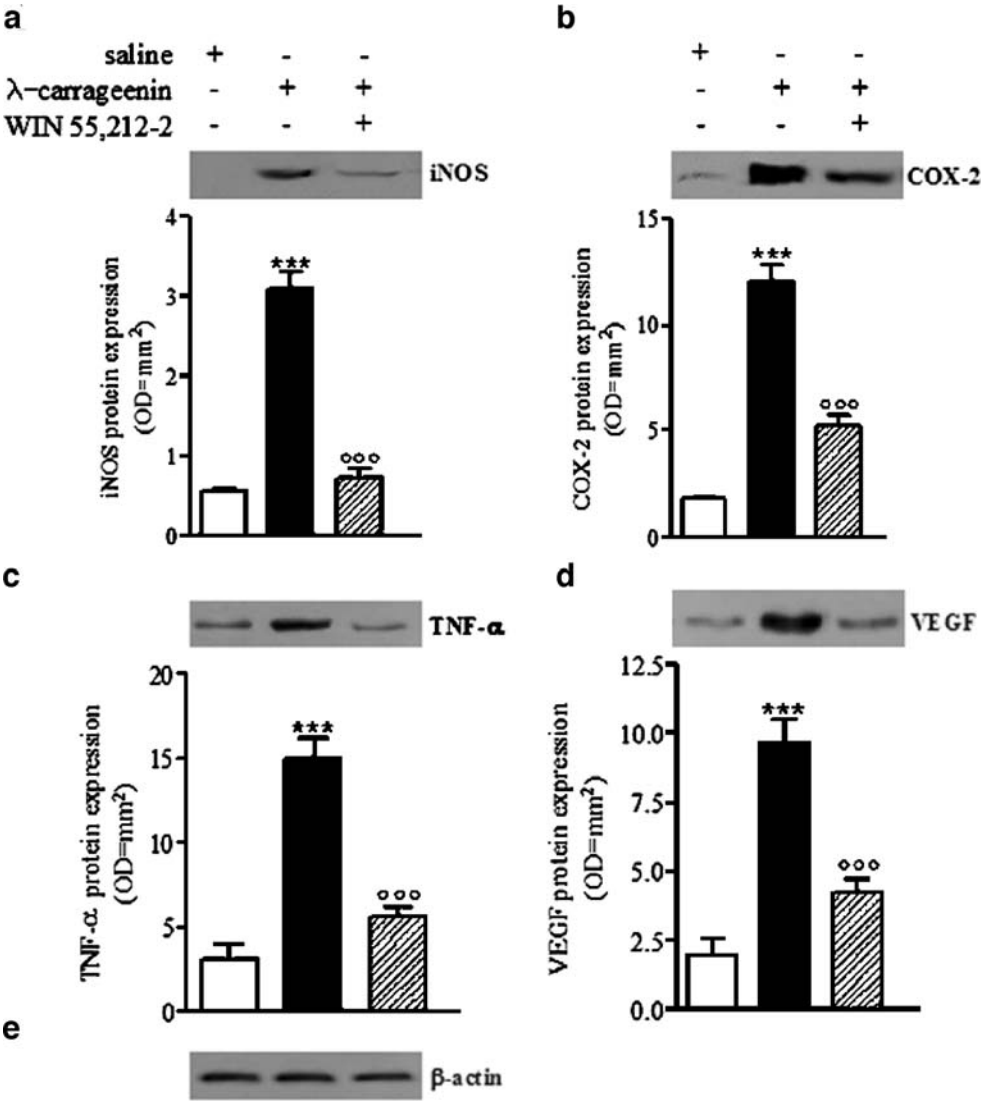

Fig. 5 Effects of WIN 55,212-2 on λ-carrageenin-induced NF-κB regulated gene expression in granulomatous tissue at 96 h. Representative Western blot analysis and relative densitometric analysis of iNOS (**a**), COX-2 (**b**), TNF-α (**c**), and VEGF (**d**). β-actin expression is shown as control (**e**). Data are representative of three separate experiments. Results are expressed as mean±SEM of three experiments. ***$p<0.001$ vs saline and °°°$p<0.001$ vs λ-carrageenin; ***$p<0.001$ vs saline and °°°$p<0.001$ vs λ-carrageenin alone

0.001, $n=24$ sponges from 12 rats), whereas it was ineffective when administered 3 and 4 days after sponge implantation (Fig. 1c). Moreover, the anti-angiogenic effect of WIN 55,212-2 given locally at time 0 was confirmed by histological analysis. Vessels formation was evaluated in paraffined sections from saline, λ-carrageenin, and WIN-55,212-2-soaked sponges by light microscopy (5.45±0.38, 13.45±0.54, 6.34±0.58 vessels; $p<0.001$; $n=15$ sections from 9 rats; Fig. 1d).

Effects of WIN 55,212-2 on λ-carrageenin-induced NF-κB activation

The granuloma formation was correlated with NF-κB activation. To detect NF-κB/DNA-binding activity, nuclear extracts from granuloma tissue were analyzed by Electrophoretic mobility shift assay (EMSA; Fig. 2a,b). A basal level of NF-κB/DNA-binding activity was detected in nuclear extracts of granuloma tissue from saline-treated sponges harvested 96 h after implant. Carrageenin induced a marked increase of NF-κB activation that was reduced by local administration of WIN 55,212-2 (0.1 mg/ml, by 54.5%, $p<0.001$, $n=16$ sponges from 8 rats) at time 0. The composition of the NF-κB complex activated by carrageenin was determined by competition and supershift experiments (Fig. 2c). The specificity of NF-κB/DNA-binding complex was demonstrated by the complete displacement of the NF-κB/DNA binding in the presence of a 50-fold molar excess of unlabeled NF-κB probe (Wild type, 50×) in the competition reaction. In contrast, a 50-fold molar excess of unlabeled mutated NF-κB probe (Mutated, 50×) or Sp-1 oligonucleotide (Sp-1, 50×) had no effect on this DNA-binding activity. The composition of the NF-κB complex activated by carrageenin was determined by using specific antibodies against p50 and p65 subunits of NF-κB proteins. The addition of either anti-p50 or anti-p65 and their combination to the binding reaction resulted in a characteristic retarded band, as well as a marked reduction

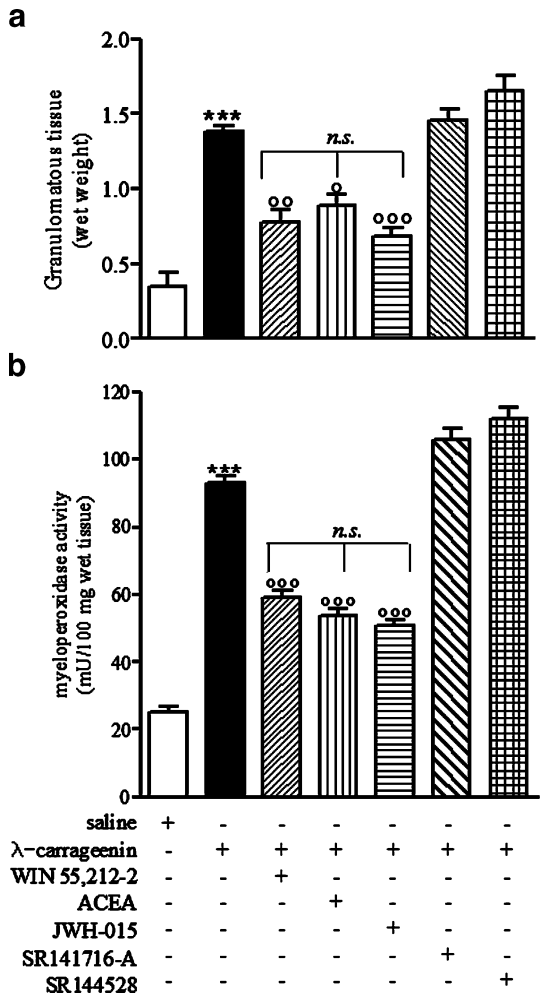

Fig. 6 Effects of ACEA (0.1 mg/ml), JWH-015 (0.1 mg/ml), SR141716-A (0.1 mg/ml), and SR144528 (0.1 mg/ml) in comparison to WIN 55,212-2 (0.1 mg/ml) on λ-carrageenin-induced granuloma formation. All cannabinoid agonists and antagonists were injected locally at the time of implantation. Granulomatous tissue formation was evaluated 96 h after implantation as wet weight of tissue around the sponge in A and as mieloperoxidase activity in B. Each *bar* shows the mean±SEM of three separate experiments. *n.s.* The difference among the different treatments is not significant. ***$p<0.001$ vs saline; °$p<0.05$, °°$p<0.01$, °°°$p<0.001$ vs λ-carrageenin alone

of NF-κB band intensity, suggesting that the NF-κB complex contained p50 and p65 heterodimers. Moreover, the presence of p50 and p65 subunits in nuclear fraction was examined by immunoblotting analysis (Fig. 3). In carrageenin-treated sponges, p50 and p65 nuclear level was increased as compared to saline. The administration of WIN 55,212-2 prevented the nuclear translocation of p50 and p65 subunits (by 68.4 and 53.1%, respectively; $p<0.001$, $n=16$ sponges from 8 rats).

Effects of WIN 55,212-2 on NF-κB-regulated gene expression

We investigated the effects of WIN 55,212-2 on the expression of several NF-κB-regulated pro-inflammatory mediators. We determined the abundance of iNOS, COX-2, VEGF, and TNF-α mRNA in the granulomatous tissue around sponge implants 96 h after treatment with saline solution, λ-carrageenin, and λ-carrageenin/WIN 55,212-2. Specifically, we subjected the total RNA to RT reaction and the resulting cDNA to semi-quantitative PCR-utilizing specific primers ("Materials and methods"). The obtained signals were quantified using Phosphor-Imager. We also amplified the same cDNA preparation with primers for β-actin mRNA as an internal control. Our results show that iNOS, COX-2, VEGF, and TNF-α transcripts were significantly increased in the granulomatous tissue around λ-carrageenin-soaked sponges, when compared to the tissue from saline-soaked sponges ($n=8$–12 sponges from 4–6 rats). The administration of WIN 55,212-2 strongly inhibited iNOS, COX-2, VEGF, and TNF-α mRNA expression compared to mRNA levels in tissue from λ-carrageenin-soaked sponges (Fig. 4). In addition, these results were confirmed by immunoblotting analysis. WIN 55,212-2 was able to significantly reduce protein amounts of COX-2, iNOS, TNF-α, and VEGF (by 56.09, 76.8, 62.5, and 55.6, respectively; $p<0.001$; $n=8$–12 sponges from 4–6 rats) induced by λ-carrageenin (Fig. 5). These findings demonstrate that WIN 55,212-2 is able to down regulate the expression of NF-κB-regulated pro-inflammatory mediators in this model of chronic inflammation.

Effects of CB receptor selective agonists and antagonists on λ-carrageenin-induced granuloma formation

ACEA (0.1 mg/ml), a CB_1 selective agonist, and JWH-015 (0.1 mg/ml), a CB_2 selective agonist, when given locally at the time of sponge implantation, were able to reduce granuloma formation (by 39.5%, $p<0.05$, $n=16$–18 sponges from 8–9 rats for ACEA; by 50.7%, $p<0.01$, $n=16$–18 sponges from 8–9 rats for JWH-015) at the same extent as WIN 55,212-2. Moreover, the effect of WIN 55,212-2 was reverted by the co-administration of SR141716-A (0.1 mg/ml), a CB_1 selective antagonist, and SR144528 (0.1 mg/ml), a CB_2 selective antagonist (Fig. 6a).

Effects of CB receptor agonists and antagonists on λ-carrageenin-induced leucocytes infiltration

Granulomatous tissue induced by λ-carrageenin was characterized by an increase in MPO activity, an indicator of PMN, compared to saline injected sponge. Local treatment at time 0 with WIN 55,212-2 (0.1 mg/ml), ACEA (0.1 mg/ml), and JWH-015 (0.1 mg/ml) significantly reduced the degree of PMN infiltration, determined as an increase in MPO activity (by 39.7, 42.2, and 45.1%, respectively; $p<0.001$, $n=16$–18 sponges from 8–9 rats). Moreover, the effect of WIN 55,212-2 was reverted by the co-administration of

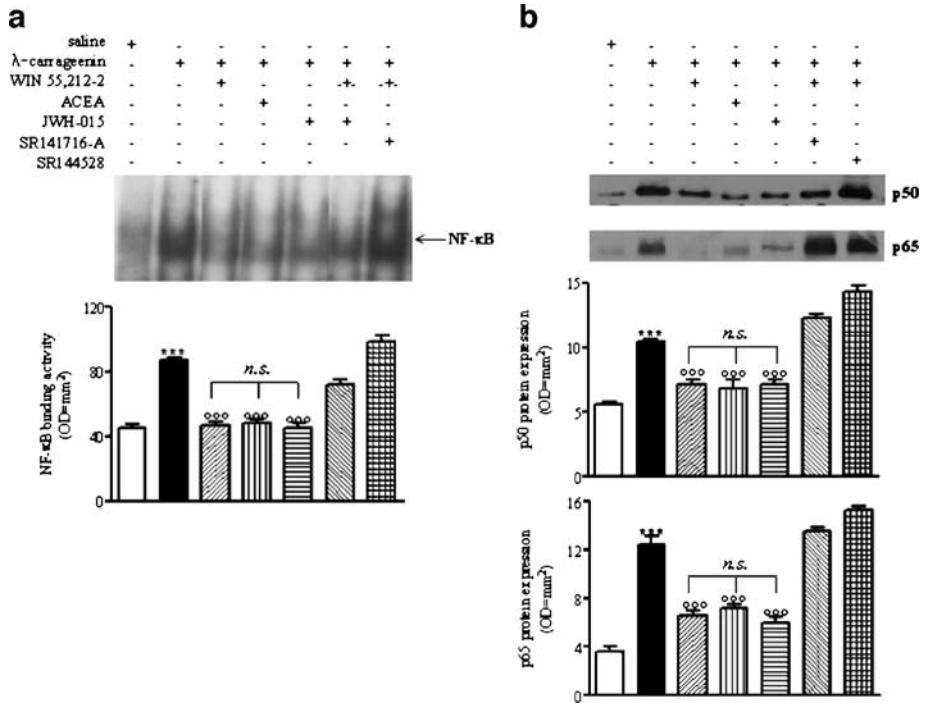

Fig. 7 Effects of cannabinoids agonists and antagonists λ-carrageenin-induced NF-κB activation in granuloma tissue at 96 h. Representative electrophoretic mobility shift assay and the relative densitometric analysis (**a**) shows the NF-κB/DNA-binding activity in nuclear extracts from granulomatous tissue. Representative Western blot and relative densitometric analysis (**b**) shows the nuclear levels of p50 and p65 in granulomatous tissue. All cannabinoid agonists and antagonists (0.1 mg/ml) were injected locally at the time of implantation. Data are representative of three separate experiments. Results are expressed as mean±SEM of $n=3$ separate experiments. *n.s.* The difference among the different treatments is not significant. ***$p<0.001$ vs saline, °°°$p<0.001$ vs λ-carrageenin alone

SR141716-A (0.1 mg/ml) and SR144528 (0.1 mg/ml; Fig. 6b).

Effects of CB receptor selective agonists and antagonists on λ-carrageenin-induced NF-κB activation

We investigated the effects of ACEA (0.1 mg/ml) and JWH-015 (0.1 mg/ml) selective agonists as well as SR141716-A (0.1 mg/ml) and SR144528 (0.1 mg/ml) antagonists on NF-κB/DNA-binding activity in nuclear extracts from granuloma tissue by EMSA (Fig. 7a). A basal level of NF-κB/DNA-binding activity was detected in nuclear extracts of granuloma tissue from saline-treated sponges harvested 96 h after implant. λ-Carrageenin induced a marked increase of NF-κB activation that was reduced by local administration of ACEA (by 51.8%, $p<0.001$, $n=16$–18 sponges from 8–9 rats) and JWH-015 (by 55.6%, $p<0.001$, $n=16$–18 sponges from 8–9 rats), as well as WIN 55,212-2, whereas SR141716-A and SR144528 reverted this effect. Moreover, the presence of p50 and p65 subunits in nuclear fraction was examined by immunoblotting analysis (Fig. 7b). In λ-carrageenin-treated sponges, p50 and p65 nuclear levels were increased as compared to saline. The administration of ACEA and JWH-015 prevented the nuclear translocation of p50 and p65 subunits (by 66.6 and 69.2%, respectively, for p50; by 48.05% and 59.9% for p65, respectively; $p<0.001$; $n=16$–18 sponges from 8–9 rats) as well as WIN 55,212-2, whereas SR141716-A and SR144528 reverted this effect. Histone-1 was used as an internal control (data not shown).

Discussion

We investigated the potential protective role of synthetic CB agonists on granuloma formation induced by λ-carrageenin-soaked sponge implant in rat. We show that daily administration of the non-selective CB_1/CB_2 receptor agonist, WIN 55,212-2, resulted in a significant reduction of granuloma formation when it was given locally, while, when WIN 55,212-2 was administered systemically, granulomatous tissue formation was only slightly and not significantly reduced. Moreover, in time-course experiments, we demonstrated that WIN 55,212-2 significantly decreased both the weight and hemoglobin content of granulomatous tissue when given at time 0, at the same extent as daily administration, but it failed to reduce the granuloma weight and hemoglobin content when given at time 3 and 4. These results suggest that WIN 55,212-2 is more effective in preventing the development of granuloma than in reducing the chronic process when it is already switched on. In this paper, we also provide evidence for the

local efficacy of WIN 55,212-2, given at time 0, in reducing leukocyte infiltration and new vessel formation induced by carrageenin in granulomatous tissue. This may be relevant, as the systemic administration of CB agonists has, as a limit, psychotropic effects due to CB_1 receptor activation in the central nervous system. Our data are in agreement with other studies demonstrating the protective effects of WIN 55,212-2 and of other synthetic CBs, as HU-210, in animal models of arthritis [26], but enlarge the study also to the new angiogenesis related to a chronic inflammatory process.

To investigate the molecular mechanism underlying WIN 55,212-2 anti-inflammatory/anti-angiogenic effect, we studied the role of WIN 55,212-2 on NF-κB activation in the granulomatous tissue. NF-κB regulates, at transcriptional level, the expression of several pro-inflammatory and pro-angiogenic genes, including those encoding for COX-2, iNOS, TNF-α, and VEGF. Our data show that WIN 55,212-2, locally injected at time 0, decreased NF-κB activation, which was correlated to a decrease in COX-2, iNOS, TNF-α and VEGF mRNA, and protein levels in granulomatous tissue.

It has been suggested that anandamide is able to inhibit TNFα-dependent activation of NF-κB by targeting both IKKβ and, to a lesser extent, IKKα, thus, blocking IκBα degradation [31]. Moreover, dexanabinol reduces NF-κB activation by inhibiting phosphorylation and degradation of IκBα [32], although the mechanism by which CBs target NF-κB remains unclear. Our results indicate that WIN 55,212-2 is able to prevent the activation of NF-κB, which, in turn, leads to the inhibition of the expression of mediators involved in the chronic inflammatory process and angiogenesis. We have previously demonstrated that, by using a decoy oligonucleotide for NF-κB, it was possible to prevent the formation of granulomatous tissue formation in rat [10]; moreover, we and other groups have shown the inhibition of NF-κB activation by both natural and synthetic CBs in in vitro models of inflammation [32, 33].

To discriminate if the anti-inflammatory effect of WIN 55,212-2 was mediated by CB_1 or CB_2 receptor agonist, we tested ACEA and JWH-015, a CB_1 and a CB_2 selective agonist, respectively, on granuloma formation. In our experimental conditions, no statistical significant difference between ACEA, JWH-015, and WIN 55,212-2 was observed in reducing granulomatous tissue formation, leukocyte infiltration, and NF-κB activation by preventing the nuclear translocation of p50 and p65 subunits. All the effects of WIN 55,212-2 were reverted by SR141716-A and SR144528, CB_1 and CB_2 selective antagonists, respectively. Therefore, our results show that the anti-inflammatory and anti-angiogenic effects of WIN 55,212-2 are mediated by both CB_1 and CB_2 receptors, through the inhibition of NF-κB activation in this chronic model of inflammation, although the molecular mechanism leading to NF-κB activation is still unclear. It is known that immune cells could express both receptors on the membrane surface; for example, mast cells, the major cell-type involved in the etiopathogenesis of granuloma formation, express both CB_1 and CB_2 receptors [34]. Furthermore, CBs could decrease the release of nitric oxide and other pro-inflammatory mediators equally through CB_1 and CB_2 receptor activation in macrophage cell lines [24]. Moreover, it has been recently reported that endogenous and exogenous CBs trigger NF-κB-dependent apoptosis in immune cells through the activation of both CB_1/CB_2 receptors [19].

In the light of the above reported data, we suggest that CB agonists prevents λ-carrageenin-induced granuloma formation and related angiogenesis through a CB_1/CB_2 receptor-dependent mechanism inhibiting NF-κB activation and NF-κB-dependent pathways.

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