Inhibitory effect of synthetic cannabinoids on cytokine production in rheumatoid fibroblast-like synoviocytes

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Abstract

Objective

To verify whether synthetic cannabinoids (CP55,940 and WIN55,212-2) are able to exert an anti-inflammatory effect on rheumatoid fibroblast-like synoviocytes (FLS) by down-regulating cytokine production, and determine whether this effect could be mediated by CB1/CB2 cannabinoid receptors.

Methods

Interleukin-6 (IL-6) and interleukin-8 (IL-8) were assayed in the supernatant from cultured FLS by ELISA method before and after 3 hours of incubation with CP55,940 (10μM) and WIN55,212-2 (10μM). Co-stimulation of cells with the cannabinoid receptor antagonists was performed to evaluate receptor involvement in cytokine modulation. All the experiments were conducted in basal conditions and after 1 hour pre-incubation with 0.1 ng/ml IL-1β.
FLS expression of CB1 and CB2 receptor was studied by Western Blot analyses.

Results

Both CP55,940 and WIN55,212-2 induced a potent and significant reduction in IL-6 and IL-8 secretion from IL-1β. stimulated FLS. Although FLS express CB1 and CB2 receptor, cannabinoid receptor antagonists did not significantly modify the inhibition of cytokines secretion induced by CP55,940 and WIN55,212-2.

Conclusions

In vitro*, CP55,940 and WIN55,212-2 exert a potent anti-inflammatory effect on rheumatoid FLS via a non-CB1/CB2 receptor mediated mechanism.*

Key words
Cannabinoids, rheumatoid arthritis, synoviocytes, cytokines.

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Introduction

Besides a group of naturally occurring derivatives of marijuana plants (*cannabis sativa*), the term "cannabinoids" includes a large group of synthetic analogues and several endogenous fatty acid derivatives with cannabinoid-like activities (endocannabinoids) (1) which are able to modulate the activities of the two cannabinoid receptors CB1 (CB1r) and CB2 (CB2r) (2, 3). However, a growing body of evidence is confirming the hypothesis that some of the biological effects of cannabinoids are mediated by additional non-classical CB1r and CB2r, involving the *Transient receptor potential vanilloid type-1* (TRPV1) (4, 5, 6) and the *family of Peroxisome Proliferator-Activated Receptors* (PPARs) (7-9).

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by progressive joint destruction. The rheumatoid pannus is a major source of proinflammatory cytokines (10-13), including IL-6 (14) and IL-8 (15), which play a pivotal role in the pathogenesis and self-maintenance of the disease. A large body of evidence has highlighted how cannabinoids reduce inflammation in animal models of arthritis. Zurier demonstrated that Ajulemic Acid (AJA), a synthetic derivative of Δ-9 tetrahydrocannabinol, reduces the severity of adjuvant-induced arthritis (16, 17) and several studies have highlighted that this anti-inflammatory property of AJA could, at least in part, be mediated by a member of the peroxisome receptors family namely PPAR-γ (18, 19). Malfait demonstrated that cannabidiol exerted a dose-dependent inhibiting activity on the development of clinical manifestations as well as on joint damage in collagen-induced arthritis (20). Sumariwalla obtained interesting results with HU-320, a metabolite of a synthetic homologue of cannabidiol, showing a reduction of joint damage in collagen-induced arthritis (21). However, the precise role of cannabinoid receptors in the modulation of the anti-inflammatory activity of cannabinoids in these experimental models of arthritis has yet to be fully elucidated.

In the light of the observations mentioned above, our study was performed with the aim of (i) evaluating whether fibroblast-like synoviocytes (FLS) express CB1 and CB2 receptors, (ii) verifying whether synthetic cannabinoids exert an anti-inflammatory effect on human rheumatoid FLS and (iii) determining whether the anti-inflammatory activity of synthetic cannabinoids is CB1r and/or CB2r-mediated. Thus, we studied the effect of two non-selective, structurally different, CB1 and CB2 receptor agonists, namely CP55, 940 and WIN55,212-2, on IL-6 and IL-8 from RA FLS in basal conditions and after IL1-β activation.

Materials and methods

Patient selection

Five patients with RA, diagnosed according to the American College of Rheumatology 1987 revised criteria, were enrolled in the study. All five patients had inflammatory knee joint involvement. Patients were considered eligible for the study if the synovial fluid leukocyte count was between 2,000 and 10,000 cells/mm^3. Patients with other possible inflammatory processes such as crystal arthritis or infection of the joints were excluded from the study. The specimens of synovial tissue were obtained during joint replacement. The control group consisted of five sex- and age-matched patients with osteoarthritis (OA) of the knee who underwent knee joint prosthesis. All patients were under treatment with steroid (mean daily dose <8 mg of prednisone-equivalent) and/or nonsteroidal anti-inflammatory drugs. None of the patients was taking disease modifying antirheumatic drugs or TNFα-blocking therapy (Table I).

Written informed consent was obtained from all patients in accordance with the Principles of the Declaration of Helsinki and the study was approved by the local research ethics committee.

Drugs and reagents

The reagents used were: human recombinant IL-1β, (Sigma-Aldrich, Italy); CB1 and CB2 receptor agonists, CP55, 940 (non classical cannabinoid lacking the dihydropyran ring of THC) and WIN55,212-2 (aminoalkylindole canna-binoid), (Tocris, UK); CB1r agonist ACEA (eicosanoid)

Table I. Demographic and clinical features of patients. Data are expressed as mean ± SD [range].

	OA	RA
Age (years)	62 ± 5 [58-71]	56 ± 8 [48-69]
Sex (no. women/no. men)	3/2	3/2
Disease duration (months)	72 ± 69 [10-110]	74 ± 38 [26-133]
DAS 28	-	4.1 ± 0.5 [3.6-5]
Synovial fluid leukocyte count (cell/mm^3)	680 ± 430 [200-1300]	5500 ± 3200 [2000-9800]
ESR (mm/1st hour)	17 ± 3 [14/23]	51.9 ± 22 [35/81]

(Tocris, UK); selective CB2r agonist JWH015 (aminoalkylindole cannabinoid); CB1 and CB2 receptor antagonists, AM281(diarylpyrazole) and AM630 (6-iodopravadoline); selective antagonist of TRPV1 capsazepine (CZP), (Tocris, UK), and PPAR-γ anta-gonist GW9662, (Alexis, CH).

Anti-human CB1 and CB2 receptors and anti-rabbit secondary antibody IgG conjugated to horseradish peroxidase were purchased from ABR (Affinity Bioreagent, USA).

Synoviocyte cultures

After sampling, synovial tissue was minced and then digested by clostridial collagenase (1 mg/ml in PBS). Cell suspensions obtained were plated out in 2 ml of DMEM supplemented with L-glutamine (2 mM), FCS (10%), penicillin (200 U/ml) and streptomycin(200 μg/ml) in 35mm/Tissue Culture Dish (35x10mm style) in humidified atmosphere containing 5% CO_2. The experiments were conducted at the 3rd passage in order to avoid changes in the original phenotype.

Cell viability

Cell viability was evaluated on FLS (plated out at a density of 1x10^4 cells/well in 96-well dishes) by using the MTT assay and trypan-blue exclusion test before and after 3 hours of treatment with CP55, 940 (10μM), WIN55,212-2 (10μM), ACEA (200nM), JWH015 (10μM), AM281 (80μM), AM630 (80μM), GW9662 (10μM), and CZP (30μM). Unless specified, GW9662, CZP and all cannabinoids were dissolved in DMSO, except of ACEA that was dissolved in ethanol. Agonists and antagonists were studied along with control solvent. Control experiments were conducted by incubating FLS with equal amounts of solvents (DMSO and ethanol). Results were expressed as mean ± standard deviation (SD) of three experiments.

Culture stimulation

FLS (1x10^5 cells) were incubated in 500μl with or without cannabinoid receptor agonists (CP55, 940 10μM, WIN55,212-2 10μM, ACEA 0.1μM and JWH015 10μM)±IL-1β (0.1ng/ml) for 3 h. Experiments with cannabinoid receptor antagonists (AM281 and AM630 80μM) and TRPV1 antagonist (CZP 30μM) were performed by incubating FLS for 10 min followed by the addition of cannabinoid agonists.

To test the PPAR-γ dependence of ligand mediated effects on synoviocytes, the irreversible PPAR-γ antagonist (GW9662 10 μM) was added to IL-1β (0.1 ng/ml) stimulated or non-stimulated FLS cultures 60 minutes prior to cannabinoid ligands.

Cytokine assay

The concentration of IL-6 and IL-8 in culture supernatants was measured by a colorimetric sandwich ELISA kit (Euroclone Lugano, CH). The minimum detectable concentration of IL-6 and IL-8 was less than 2 pg/ml.

Immunoblot analysis

Equal amounts of protein were separated from samples by sodium dodecyl sulphate (SDS)- PAGE on a 12% bis/acrylammide gel and the protein was transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, VA). The membrane was blocked with 5% non-fat milk and 1% bovine serum albumin for 1 h and incubated with polyclonal anti-CB1r (1:200) and polyclonal anti-CB2r (1:200) primary

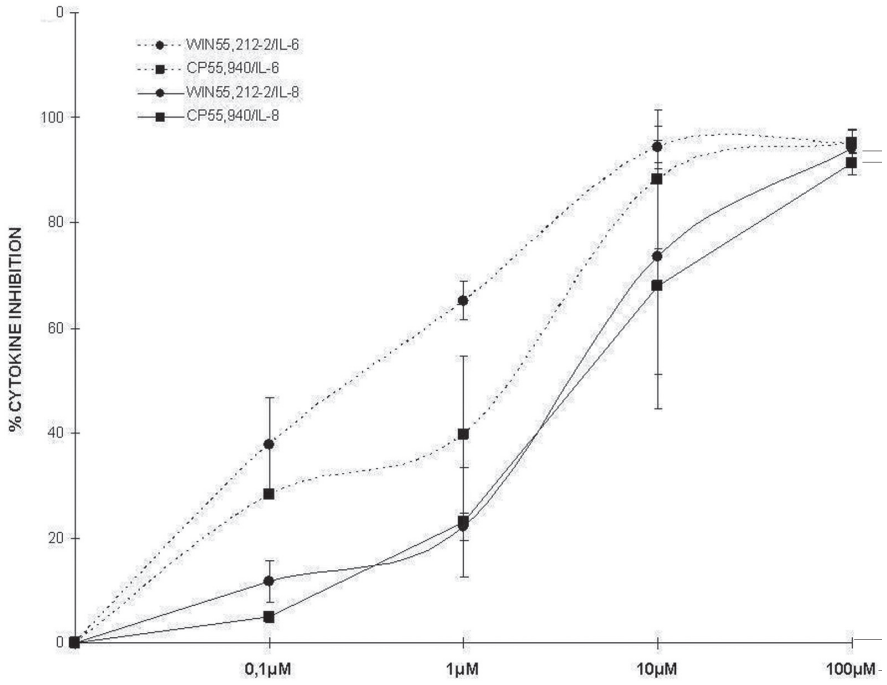

Fig. 1. Effect of CP55,940 and WIN55,212-2 on IL-6 and IL-8 levels released by IL-1β activated RA FLS. Cells were incubated for 3 h in the presence of increasing concentrations (0.1, 1, 10, 100 μM) of CP55,940 and WIN55,212-2. Cytokine levels showed a significant dose-dependent reduction. IL-6 and IL-8 were measured by ELISA as described in the methods section. Results are expressed as means ± SD of five separate experiments.
(p<0.05; Fisher's F and Hotelling's Test).

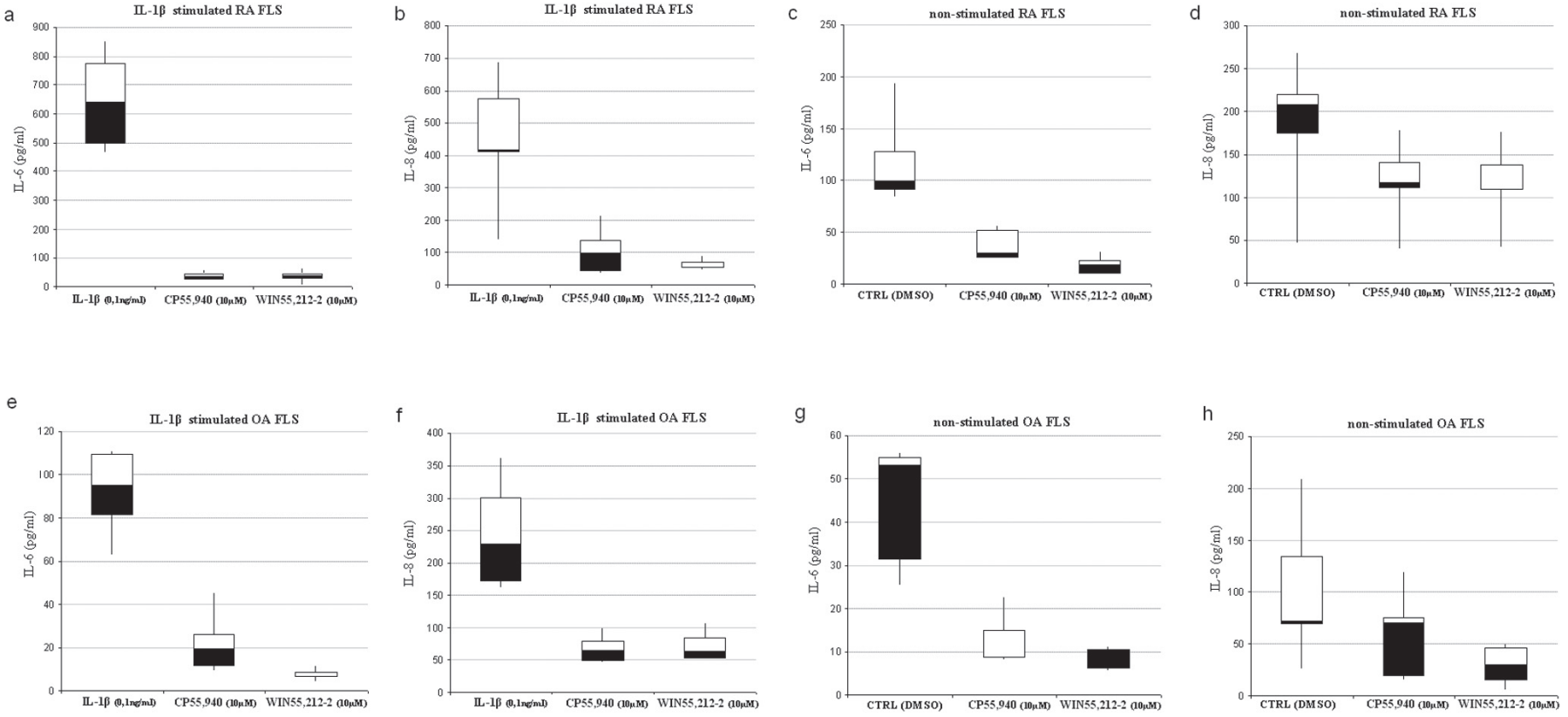

Fig. 2. Effects of CP55,940 (10 μM) and WIN55,212-2 (10 μM) on supernatant levels of IL-6 and IL-8 from IL1-β stimulated and non-stimulated RA FLS **(a),(b),(c),(d)** and OA FLS **(e),(f),(g),(h)**. CP55,940 (10 μM) and WIN55,212-2 (10 μM) induced a significant reduction of IL-6 in all groups studied ($p<0.05$; Kruskal-Wallis on ranks), whereas IL-8 was significant reduction in IL1-β stimulated RA and OA FLS ($p<0.05$; Kruskal-Wallis on ranks), but not in non-stimulated RA and OA FLS.
IL-6 and IL-8 were measured by ELISA as described in the methods section. Results are expressed as medians and quartile charts (n=5).

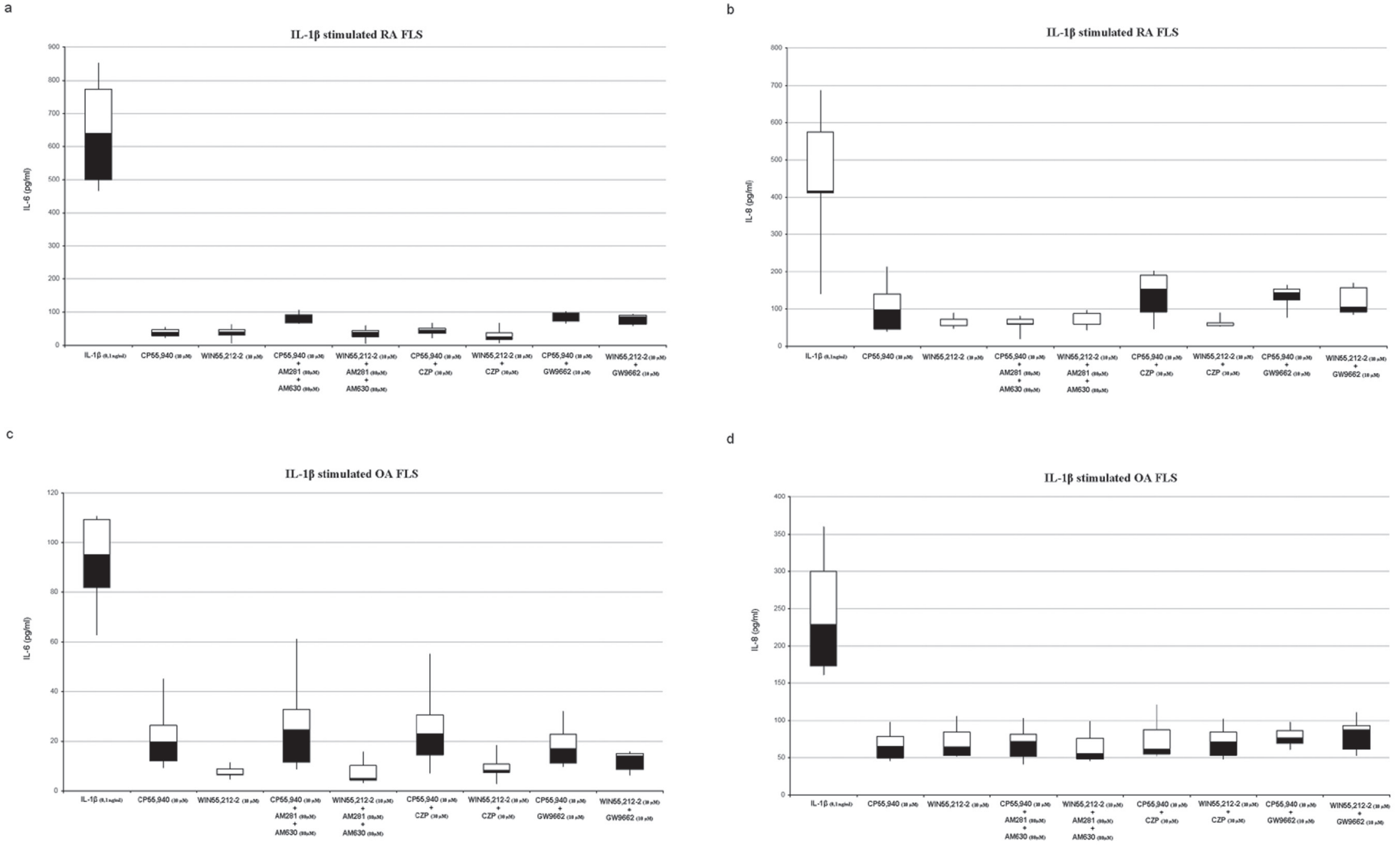

Fig. 3. Effects of CP55,940 (10 μM) and WIN55,212-2 (10 μM) on supernatant levels of IL-6 and IL-8 from IL-1β stimulated RA **(a),(b)** and OA **(c),(d)** FLS pre-treated with AM281 (80 μM), AM630 (80 μM), CZP (30 μM) and GW9662 (10 μM). Cells were pre-treated with the selective CB1r and CB2r antagonists (AM281 and AM630), with the selective TPRV1 antagonist (CZP) and with PPAR-γ antagonist (GW9662) prior to incubation with CP55,940 or WIN55,212-2 for 3 hours. CP55,940 (10 μM) and WIN55,212-2 (10 μM) induced a significant reduction in IL-6 and IL-8 levels in all groups ($p<0.05$; Kruskal-Wallis on ranks). IL-6 and IL-8 were measured by ELISA as described in the *Methods* section. Results are expressed as medians and quartile charts (n=5).

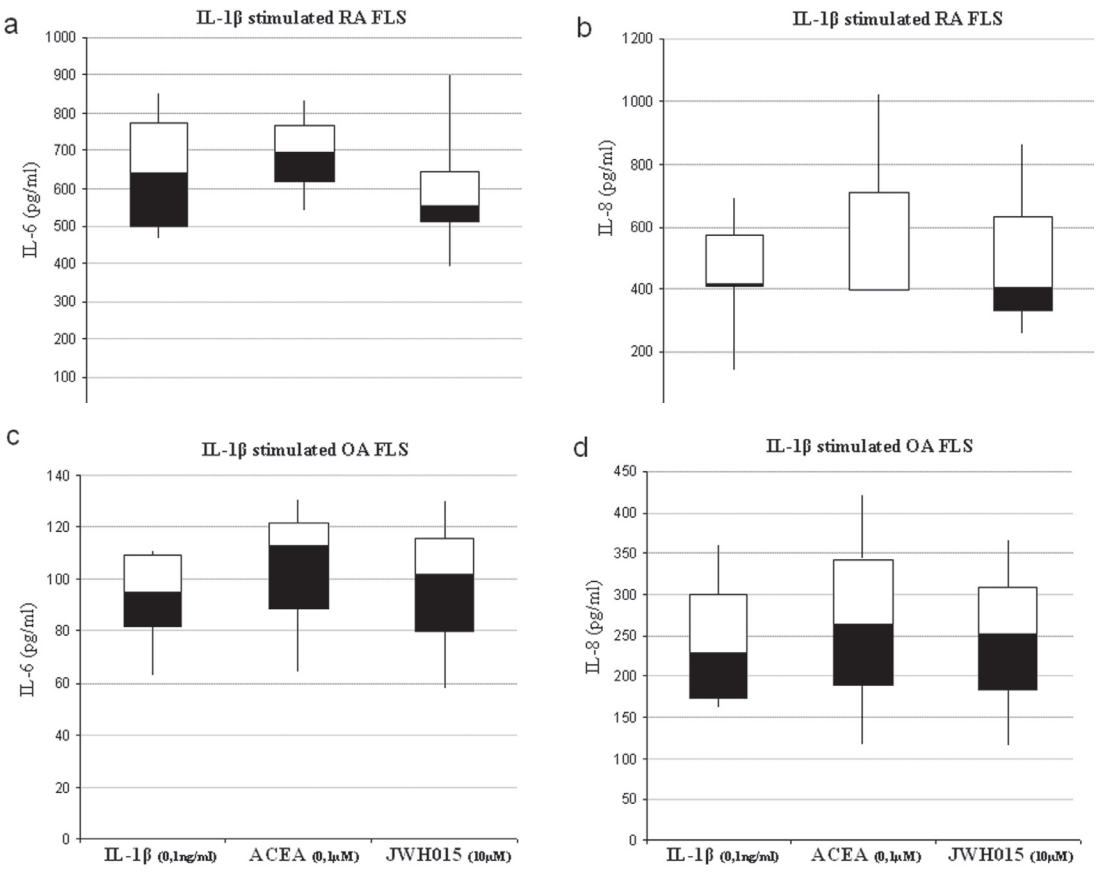

Fig. 4. Effects of the selective CB1r agonist, ACEA (0.1 μM), and selective CB2r agonist, JWH015 (10 μM), on IL-6 and IL-8 released from IL-1β stimulated RA **(a),(b)** and OA **(c),(d)** FLS. No significant differences were found in any groups.
IL-6 and IL-8 were measured by ELISA as described in the methods section. Results are expressed as medians and quartile charts (n=5).

antibodies for 2 hours at room temperature. After washing, the membrane was incubated with donkey anti-rabbit secondary antibody IgG conjugated to horseradish peroxidase (1:2500) for 45 minutes and subjected to chemiluminescence detection. β-actin (45kDa) was used as control protein.

Statistical analysis

For the comparison of dose-response curves, Fisher's F-test and Hotelling's trace statistics were used to test monovariate and multivariate effects, respectively. The inter-group comparison was performed by the Kruskall-Wallis Test on Ranks followed by Dunnett's post-hoc test. A statistical significance level of 95% (p<0.05) was used. All data analyses were performed using the SPSS statistical package.

Results

To determine whether cytokine production could be influenced by synthetic cannabinoids, OA and RA FLS were incubated with CP55,940 and WIN55,212-2. CP55,940 and WIN-55,212-2 were not toxic at the concentrations used in our experiments, viability being always >86±3% (p<0,05).

On the basis of a time-dependent cytokine decrease, the incubation time of cannabinoids was established at 3 h (data not shown). The level of cytokines, assayed in supernatant of RA FLS, after 3 h of incubation with CP55,940 and WIN55,212-2, showed a dose-dependent reduction in IL1-β induced IL-6 and IL-8 release, with a significant linear fitting (p<0.05; Fisher's F and Hotelling's Test) (Fig. 1). The experiments were conducted with CP55,940 and WIN55,212-2 at 10 μM being the most effective dosage according to dose response curve.

An MTT assay confirmed good cell vitality as 96±5% FLS were metabolically active at concentrations of 10μM. Similarly, neither the cannabinoid selective agonists nor the selective antagonists showed any significant toxicity at the concentrations used in this study. As expected, control experiments run with same amount of ethanol or DMSO did not significantly alter cell viability.

In IL1-β stimulated RA FLS, CP55,940 (10μM) and WIN55,212-2 (10μM) induced a significant mean reduction of 91% in IL-6 supernatant levels and 71% in IL-8 supernatant levels.

The percentage of inhibition induced by CP55,940 (10 μM) and WIN55,212-2 (10 μM) was observed not to be significantly different between OA and RA FLS. CP55,940 (10 μM) and WIN55,212-2 (10 μM) promoted a significant mean reduction of 77% in IL-6 supernatant levels from non-stimulated RA FLS, as well as of 58% in IL-6 supernatant levels from non-stimulated OA FLS. By constrast, cannabinoid agonists did not significantly reduced IL-8 supernatant levels from non-stimulated OA and RA FLS (Fig. 2).

To further investigate whether the cyto-kine downregulation was mediated, at least in part, by CB1r or CB2r, cell cultures were preincubated with the selective CB1r antagonist AM-281 (80 μM) and the CB2r antagonist AM-630 (80 μM). These antagonists did not significantly modify the inhibition of IL-6 and IL-8 release promoted by CP55,940 (10 μM) and WIN55,212-2

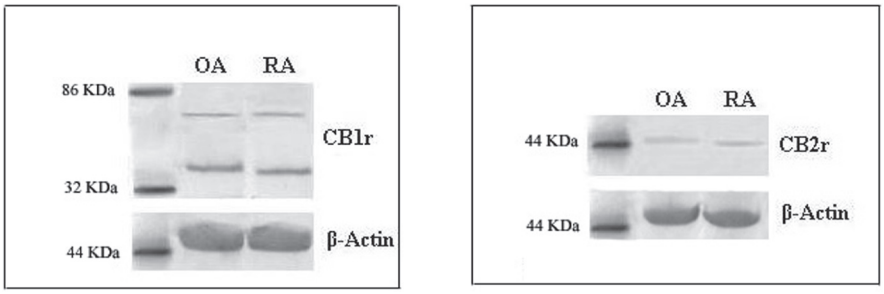

Fig. 5. CB1r and CB2r expression by human FLS from RA and OA. Whole cell lysates were analyzed by Western Blotting using specific antibodies against CB1r, CB2r and control protein β-actin. CB1r is represented by the 60 kDa and by the post-transcriptional isoform of 37 kDa bands **(a)**; CB2r protein is expressed by the 45 kDa band **(b)**.

(10 μM) (Fig. 4). Furthermore, the incubation of OA and RA FLS with the CB1r agonist ACEA (0.1 μM) and the CB2r agonist JWH-015 (10 μM) did not significantly affect IL-6 and IL-8 levels, confirming a CB1r-CB2r independent mechanism (Fig. 4).

Moving on from these observations, we evaluated the possibility of a TRPV1-mediated effect by using the competitive antagonist capsazepine (CZP) (30 μM) in all the groups studied. CZP did not significantly revert the inhibitory effect of synthetic cannabinoids. Similarly, the pre-incubation of FLS with PPAR-γ antagonist GW9662 (10 μM) did not suppress the cytokine inhibition induced by CP55,940 (10μM) and WIN55,212-2 (10μM), thus indirectly indicating a PPAR-γ-independent mechanism (Fig. 3).

In order to verify whether FLS express cannabinoid receptors, Western blotting was carried out on non-stimulated OA and RA FLS. In all the groups we observed two immunoreactive bands for CB1r migrating at around 60 and 37 kDa (22), as well as a 45 kDa band corresponding to the CB2r protein (Fig. 5).

Discussion

We have demonstrated that CP55,940 and WIN55,212-2 potently reduce supernatant levels of IL-6 and IL-8 released by IL-1β stimulated FLS. The inhibitory effect on cytokine production was also observed for IL-6, but not IL-8, in non-stimulated synoviocytes.

Our experiments, which focused on selective agonism-antagonism for CB1r and CB2r, indicate that the inhibitory effect of cannabinoids on cytokine production was not mediated by such receptors, although we have demonstrated the expression of CB1r and CB2r in FLS. A growing body of literature describes the immunomodulatory and anti-inflammatory activities of cannabinoids (6). Endocannabinoids released by macrophages, dendritic cells and lymphocytes are likely to be involved in innate and adaptive immunity, modulating cell migration, cytokine production, and chemotaxis. It has been demonstrated *in vitro* that CP55,940 decreases macrophage NO production (23) and macrophage migration (24), and inhibits T-helper, T cytotoxic, and NK activity (25). WIN55,212-2 has been described to reduce NO production and proteoglycan degradation in bovine chondrocytes (26), inhibit inflammatory mediators (including TNF-α, CXCL10, CCL2, and CCL5) in human foetal astrocytes (27), and may modulate IL-8 release by negatively regulating the signalling cascade leading to the activation of NF-κB (28). Previous in vivo observations concerning the antiarthritic activity of cannabidiol (20,29), ajulemic acid (19, 30), and HU320 (21, 31) support our *in vitro* observations that WIN55,212-2 and CP55,940 potently and dose-dependently down-regulate IL-6 and IL-8 production, independently of their structural differences.

In contrast to previous observations (26-28), on the role of incubation time our data showed that cannabinoids are able to exert their biologic effects rapidly, thereby promoting the maximal cytokine down-regulation in few hours. Peculiarly, the IL-8 level did not significantly decrease after cannabinoid exposure in non-stimulated FLS, suggesting that longer incubation times are required, which is consistent with the time-dependent activation of cellular transduction pathways (28).

Although WIN55,212-2 and CP55,940 are high affinity CB1r and CB2r agonists, we did not observe any change in their inhibitory effect on cytokine production when FLS were preincubated with the selective CB1r antagonist AM-281 and the selective CB2r antagonist AM-630, probably excluding a CB1r or CB2r involvement. Similarly, no effect on cytokine production was found by incubating FLS with the selective CB1r agonist ACEA or the selective CB2r agonist JWH-015, which is in a way consistent with our hypothesis of a lack of a CB1r or CB2r mediated mechanism.

Taken together, these data support the recent view that a third way of action should be postulated. Nevertheless, in our experimental model, the blockade of additional putative mediators of several cannabinoid activities, including PPAR-γ and TRPV1 receptors, did not affect the robust anti-inflammatory activity of cannabinoids.

At present some of the biological activities of cannabinoids cannot be fully explained by CB1r/CB2r mediated mechanisms, which is consistent with the hypothesis that additional – as yet unidentified – receptorial and non-receptorial pathways could be involved in signal transduction (32). Several studies agree that some immunomodulatory effects of cannabinoids are not mediated by receptors and it is conceivable that "non-specific binding sites" are responsible for some of the biologic properties of cannabinoids that seem likely not to be receptorial (33). Indeed, in addition to well-defined cannabinoid receptor-coupled signalling pathways, more recent data have provided evidence that other transducing systems may be involved in cannabinoid effects by down-regulating two signalling pathways including the extracellular signal-related kinases (ERK-1 and ERK-2) and survival pathways (Akt) (34) that are critical for the signalling of various extracellular stimuli such as proinflammatory cytokines (35).

In conclusion, it is conceivable that several cannabinoids share intrinsic anti-

inflammatory activity independently of their structural differences. Further studies are necessary to support the intriguing hypothesis that would indicate cannabinoids as a promising pharmacological tool for the treatment of inflammatory rheumatic disorders (36).

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