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Effects of cannabinoids on nitric oxide production by chondrocytes and proteoglycan degradation in cartilage

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Abstract

Cannabinoids have been reported to have anti-inflammatory effects and reduce joint damage in animal models of arthritis. This suggests a potential therapeutic role in arthritis of this group of compounds. Cannabinoids were studied to determine whether they have direct effects on chondrocyte metabolism resulting in cartilage protection. Synthetic cannabinoids, *R*-(+)-Win-55,212 (Win-2) and *S*-(−)-Win-55,212 (Win-3) and the endocannabinoid, anandamide, were investigated on unstimulated or IL-1-stimulated nitric oxide (NO) production in bovine articular chondrocytes as well as on cartilage proteoglycan breakdown in bovine nasal cartilage explants. Win-2 significantly inhibited ($P < 0.05$) NO production in chondrocytes at 1–10 μM concentrations. The combined CB_1 and CB_2 cannabinoid receptor antagonists, AM281 and AM630, respectively, at 100 μM did not block this effect, but instead they potentiated it. Anandamide and Win-2 (5–50 μM) also inhibited the release of sulphated glycosaminoglycans in bovine cartilage explants. The results suggest that some cannabinoids may prevent cartilage resorption, in part, by inhibiting cytokine-induced NO production by chondrocytes and also by inhibiting proteoglycan degradation.

Keywords: Cannabinoids; Cartilage resorption; Chondrocytes; Collagen; Interleukin-1α; Proteoglycan

1. Introduction

Cannabis has been used for medicinal purposes for thousands of years. Over 60 different compounds have been identified in *Cannabis sativa*, collectively known as cannabinoids [1]. The best known cannabinoid is Δ^9-tetrahydrocannabinol (THC), the main psychoactive component of cannabis [2]. Cannabinoids produce their effects by acting through membrane-bound receptors, and there is also evidence of non-receptor-mediated mechanisms [3]. Two cannabinoid receptors have been identified and cloned to date, referred to as CB_1 and CB_2 cannabinoid receptors, found mainly in the central and peripheral nervous system and immune system, respectively [4–6]. There are indications that other family members may exist [7]. The CB_1 and CB_2 cannabinoid receptors are members of the seven-transmembrane superfamily of receptors, coupled to $G_{i/o}$ proteins [8]. A number of endogenous cannabinoid receptor ligands (endocannabinoids) have also been identified to date, the best known being arachidonylethanolamide (AEA) also known as anandamide, 2-arachidonylglycerol (2-AG) and palmitoylethanolamide (PEA). Apart from the plant-derived cannabinoids and endocannabinoids, a number of their synthetic analogues have been developed and are available commercially including CB_1 and CB_2 cannabinoid receptor antagonists.

Cannabinoids have been reported to have analgesic, anti-inflammatory and immunosuppressive effects in animal models of arthritis [9,10]. Degradation of cartilage is a central pathological feature of diseases such as

Abbreviations: AM281, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)-methanone; anandamide, *N*-(2-hydroxyethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; IL-1α, interleukin-1 alpha; NO, nitric oxide; PBS, phosphate buffered saline; sGAG, sulphated glycosaminoglycan; Win-2, *R*-(+)-Win-55,212 [*R*-(+)-[2,3-dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate]; Win-3, *S*-(−)-Win-55,212 [S-(−)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate]

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osteoarthritis and rheumatoid arthritis. Chondrocytes maintain cartilage tissue homeostasis, sustaining the crucial balance between the rate of biosynthesis and incorporation of matrix components, and the rate of their degradation and subsequent loss from the cartilage into the synovial fluid [11]. Active proteinases such as aggrecanases, collagenases and matrix metalloproteinases (MMPs) are involved in cartilage resorption. They degrade major components of cartilage extracellular matrix, collagen (mainly type II collagen) and proteoglycans (mainly aggrecan) [12]. These proteinases are secreted from the cells in a latent form, requiring activation extracellularly, and are inhibited by tissue inhibitors of metalloproteinases (TIMPs) [11]. In rheumatic conditions such as osteoarthritis and rheumatoid arthritis there is an imbalance between the proteinases and TIMPs that accounts at least in part for the observed cartilage destruction [11,12].

Proteoglycan breakdown is an early event in cartilage degradation, while significant catabolism of collagen occurs later and this may represent the point of irreversible cartilage damage [13]. Cytokines such as interleukin-1 (IL-1), IL-6, oncostatin M (OSM), tumour necrosis factor (TNF-α) and IL-17 have been reported to induce cartilage breakdown [14–16], whilst protective effects against cartilage breakdown were shown by IL-4, IL-13, transforming growth factor-β1 (TGF-β1) and insulin-like growth factor-1 (IGF-1) [17–19]. IL-1 is a key inflammatory mediator by which the chondrocytes and synoviocytes enhance their protease production [12]. Chondrocytes produce large quantities of nitric oxide (NO) from L-arginine oxidation by inducible nitric oxide synthase (iNOS or NOS-II), when stimulated by IL-1 or lipopolysaccharide (LPS) [20]. Also, cartilage obtained from arthritic patients produces significant amounts of NO ex vivo, even in the absence of IL-1 or LPS [20]. NO derived from iNOS has been implicated in tissue injury in a variety of pathological conditions [20,21] and there is increasing evidence that excess NO production could be implicated as a factor in the aetiopathogenesis of osteoarthritis [22–24]. Inhibition of NO production then could be a useful target in the search for possible treatments for arthritis. The present study investigated whether cannabinoids have effects on chondrocyte metabolism so leading to reduced cartilage breakdown, particularly that involving cytokine-induced NO production and matrix degradation. These studies may form the basis for the identification of novel potential anti-arthritic drugs.

2. Materials and methods

2.1. Cannabinoids

The endogenous cannabinoid, anandamide, the synthetic cannabinoids [Win-2 and Win-3] and the antagonists [AM281 and AM630], that are also inverse agonists of cannabinoid receptors CB_1 and CB_2, respectively, were studied along with their solvent controls. Absolute ethanol (0.1% final concentration) was used as the solvent for anandamide and dimethyl sulphoxide (DMSO) (0.05–0.5% final concentration) was used as the solvent for the other cannabinoids. The synthetic cannabinoids were purchased from Sigma–Aldrich, and the rest of the cannabinoids were purchased from Tocris. Their effects were investigated on unstimulated or IL-1α-stimulated chondrocyte NO production and cartilage proteoglycan breakdown at concentrations which were demonstrated to be non-toxic to chondrocytes. IL-1α was purchased from the National Institute for Biological Standards and Control (NIBSC).

2.2. Bovine chondrocyte culture

Chondrocytes were obtained from bovine metacarpophalangeal joints by sequential enzymatic digestion of the cartilage pieces and plated into 24-well plates as described in [25]. Briefly, cartilage pieces were sliced from the articular joint and washed in PBS (GIBCO). The pieces were then digested in 0.25% trypsin (GIBCO) for 30 min at 37 °C followed by a wash in DMEM [supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml) plus 10% heat-inactivated FBS] (complete medium) and further digested at 37 °C overnight in sterile filtered 3 mg/ml collagenase type 1 (Sigma–Aldrich) dissolved in complete medium. The medium and the supplements were purchased from GIBCO. The cells were washed in PBS and centrifuged at 200 × *g* for 7 min. The chondrocytes were plated into 24-well plates at 2×10^5 cells/cm^2 in complete medium. The cells were cultured in a humidified incubator at 37 °C in an atmosphere of 5% CO_2. After 5 days, chondrocytes had attached to the surface, were less than 80% confluent. They were incubated with or without cannabinoids ± IL-1α (100 U/ml ≡ 0.06 nM) for 48 h in DMEM with the supplements except FBS.

2.3. Cytotoxicity studies

The methylthiazol tetrazolium (MTT) assay (Sigma-Aldrich) was used to determine any effects of anandamide and Win-2 on the cell viability of cultured chondrocytes [26]. The lactate dehydrogenase (LDH) release assay kit (Sigma–Aldrich) was also used to determine the effects of cannabinoids on cell-membrane integrity in cultured cartilage explants.

2.4. NO determination

NO was measured as nitrite in chondrocyte culture media using the modified Griess reagent (Sigma–Aldrich) [27]. Absorbance was measured at 550 nm. Nitrite concentration was then determined from a sodium nitrite standard curve (0–100 μM).

2.5. Cartilage proteoglycan breakdown

This was determined using cultured bovine nasal cartilage explants that were either unstimulated or stimulated to resorb with IL-1α (500 U/ml ≡ 0.3 nM) in the presence or absence of cannabinoids (5–100 μM) for 4 days in DMEM without FBS. The cartilage explants were prepared as described in [28]. Briefly, the bovine nasal septum was dissected from a bovine nose, cleaned of mucous membrane and washed in PBS. The nasal cartilage was sliced (about 3 mm thick slices) and discs (3 mm in diameter) were prepared using a sterilized leather punch. The explants were washed in PBS and were incubated for at least 24 h in DMEM with 10% FBS prior to the 4-day incubation. Proteoglycan release was determined as sGAG in the conditioned culture media using the dimethylmethylene blue assay [29]. The residual cartilage explants were digested using 25 mg/ml papain in 0.1 M phosphate buffer (pH 6.5) containing 50 mM disodium EDTA and 50 mM cysteine hydrochloride for 2 h at 65 °C. The sGAGs in the digests were measured as in the conditioned media. The absorbance was read at 520 nm. A standard curve (0–40 μg/ml) was constructed using shark chondroitin sulphate (Sigma–Aldrich). Proteoglycan breakdown was expressed as a percentage of total sGAGs released.

2.6. Statistical analysis

All data have been presented as mean ± s.e.m. Statistical differences from appropriate controls were determined using Kruskal–Wallis non-parametric test followed by Dunn's post-test [for data not following the Gaussian distribution, determined by Kolmogorov–Smirnov (KS) normality test] or one-way ANOVA followed by Boniferroni's post-test. In both a probability level of $P < 0.05$ was considered statistically significant. The GraphPad Prism statistical package was used (GraphPad Software Inc.).

3. Results

3.1. Cytotoxicity studies

The cytotoxicity studies showed that the cannabinoids may be toxic to bovine chondrocytes at concentrations greater than 100 μM. The antagonists used did not induce cell death even at concentration of up to 100 μM. No toxic effects of the cannabinoids, at concentrations up to 100 μM, were detected on bovine cartilage explants.

3.2. Effect of cannabinoids on cytokine-induced NO production

3.2.1. Effect of Win-2 alone or in combination with AM281

Treatment of bovine articular chondrocytes with IL-1α (100 U/ml) significantly increased NO production in these experiments. Win-2 at concentrations 0.5–10 μM was used to study the effect of synthetic cannabinoids on IL-1α-induced NO production (Fig. 1). Win-2 1–10 μM reduced the basal level of NO production whilst at 0.5 μM increased the basal level of NO production (Fig. 1). In IL-1α-treated chondrocytes, 0.5 μM Win-2 did not have any significant effect but at 1–10 μM it significantly ($P < 0.001$) reduced NO production compared with the respective vehicle controls, while 5–10 μM reduced NO production to basal levels. Chondrocytes were also treated with 100 μM AM281, an antagonist/inverse agonist of the CB1 receptor, alone or in the presence of Win-2 and stimulated with IL-1α or unstimulated (Fig. 1). AM281 did not have any effect on unstimulated chondrocytes but significantly reduced NO production in IL-1-stimulated chondrocytes compared with the vehicle controls ($P < 0.001$). Treatment of IL-1-stimulated chondrocytes with a combination of Win-2 and AM281, further reduced the NO production, below the effect of each component alone, to the extent that even 0.5 μM Win-2 in combination with AM281 reduced NO production to the basal level.

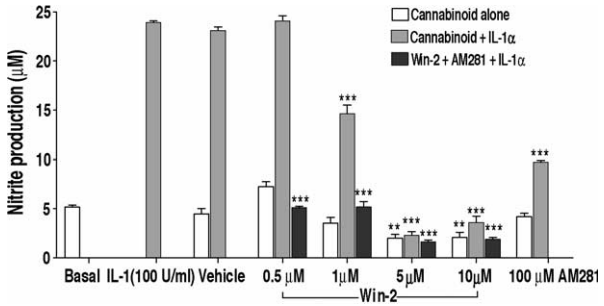

Fig. 1. Effects of Win-2 (0.5–10 μM) with or without CB_1 cannabinoid receptor antagonist, AM281 (100 μM), on IL-1α-induced NO production in bovine articular chondrocytes. Results presented as mean ± s.e.m. ($n = 6$); ** $P < 0.01$ and *** $P < 0.001$ compared with appropriate vehicle controls.

3.2.2. Effect of Win-2 alone or in combination with AM630

Win-2 (1 and 10 μM) and 100 μM AM630 with or without IL-1α (100 U/ml) were used in these experiments (Fig. 2). In unstimulated chondrocytes, Win-2, at both concentrations reduced NO production to basal levels while AM630 also reduced NO production to near basal level. When the chondrocytes were stimulated with IL-1α, Win-2 at both concentrations significantly reduced NO production, and 10 μM Win-2 reduced NO production to the basal levels. AM630, alone, also reduced IL-1-induced NO production significantly compared with its vehicle control. 1 μM Win-2 combined with AM630, further reduced NO production, but this reduction was not as marked as that with AM281. The 10 μM Win-2 in combination with AM630 also reduced NO production significantly ($P < 0.001$) compared with vehicle control.

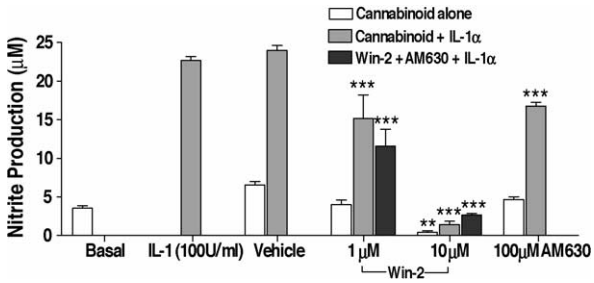

Fig. 2. Effects of Win-2 (1–10 μM) with or without CB_2 cannabinoid receptor antagonist AM630 on IL-1α-induced NO production in bovine articular chondrocytes. Results presented as mean ± s.e.m. ($n = 6$); ** $P < 0.01$ and *** $P < 0.001$ compared with appropriate vehicle controls.

3.2.3. Effect of Win-3 compared with Win-2

The inactive cannabinoid receptor enantiomer, Win-3 did not have any significant effect on IL-1α-induced NO production (Fig. 3). Win-2 5 and 10 μM, however, reduced IL-1α-induced NO production to basal levels. This effect was significantly different compared with the vehicle controls ($P < 0.001$).

3.2.4. Effect of anandamide

Anandamide (10 μM) did not show any significant effect on IL-1-induced NO production in bovine articular chondrocytes (Fig. 4).

3.3. Effect of anandamide and Win-2 on cytokine-induced proteoglycan degradation

Treatment of bovine nasal cartilage explants for 4 days with IL-1α (500 U/ml) increased sGAG release, by approximately 30% compared with the unstimulated explants. Anandamide 10 and 50 μM significantly ($P < 0.05$ and $P < 0.01$, respectively) reduced the IL-1-induced release of sGAGs compared with the vehicle controls (Fig. 5a). Win-2, 5–50 μM also inhibited IL-1α-stimulated sGAG release, reducing the sGAG release to basal levels, at all concentrations used (Fig. 5b). Win-2 appeared more potent than anandamide in preventing sGAG release.

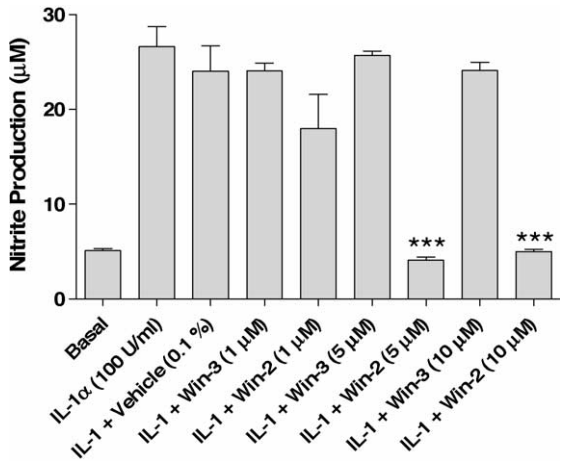

Fig. 3. The Effect of Win-2 (active stereoisomer) and Win-3 (inactive stereoisomer) on IL-1α-induced NO production in bovine articular chondrocytes. Results presented as mean ± s.e.m. ($n = 6$); *** $P < 0.001$ compared to vehicle controls.

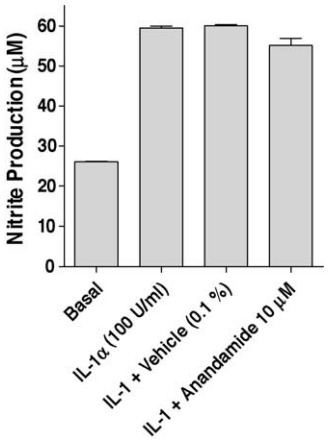

Fig. 4. The effect of anandamide on IL-1α-induced NO production in bovine articular chondrocytes; anandamide 10 μM was used in this experiment. Results presented as mean ± s.e.m. ($n = 6$).

4. Discussion

The results show that at micromolar concentrations, which are not cytotoxic, synthetic cannabinoid, Win-2 inhibits IL-1α-induced NO production and both anandamide and Win-2 inhibit IL-1α-induced proteoglycan degradation in bovine articular chondrocytes and nasal cartilage explants, respectively. In all the studies Win-2 appeared more potent compared with that of anandamide. Anandamide may be less efficacious in some of the assays possibly because it is readily metabolized by the fatty acid amide hydrolase (FAAH).

The inverse agonists, AM281 and AM630, showed some unexpected effects on NO production in bovine articular chondrocytes. When applied alone, they would be expected to enhance NO production if their action was through the cannabinoid receptors but instead they reduced NO production. Also instead of inhibiting the effect of Win-2 on NO production they appeared to act synergistically when applied in combination with the cannabinoid. This implies that AM281 and AM630, in this case were not acting through the respective CB_1 or CB_2 cannabinoid receptors. However, AM630 besides being an inverse agonist at CB_2 receptors it also behaves as a weak partial agonist at CB_1 receptors [30]. Therefore it is possible that AM630 in this study was also acting as a partial agonist at the CB_1 cannabinoid receptor at the concentration used.

It is also possible that these inverse agonists were acting via receptors other than the cannabinoid receptors or their effects were receptor independent. From these results, it is not possible to tell whether the effect of Win-2 was

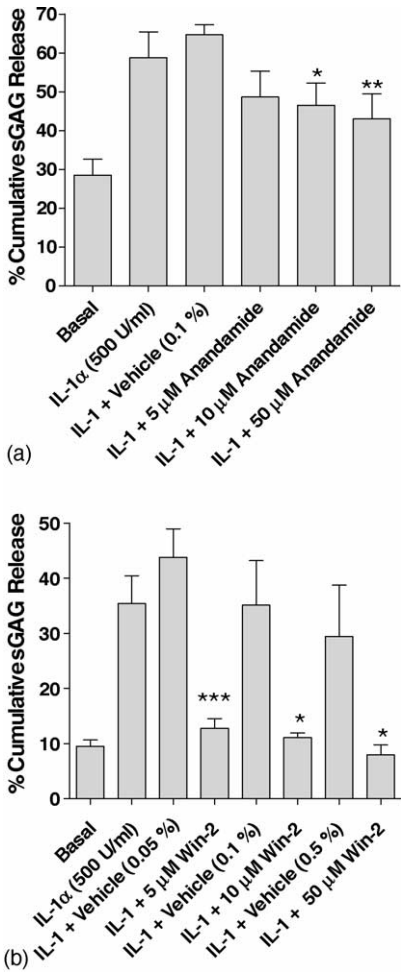

Fig. 5. (a) Effects of anandamide (5–50 μM) on IL-1α-induced GAGs release in bovine nasal cartilage explants. Results expressed as mean ± s.e.m. ($n = 6$); $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with vehicle control. (b) Effects of Win-2 (5–50 μM) on IL-1α-induced GAGs release in bovine nasal cartilage explants. Results expressed as mean ± s.e.m. ($n = 6$). $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with appropriate vehicle control (5, 10 and 50 μM compared with 0.05, 0.1 and 0.5% DMSO, respectively. Stock solution concentration was so low that it was impossible to maintain vehicle concentration to at least 0.1% for all treatments).

mediated through either CB_1 or CB_2 cannabinoid receptors. However, in the study where Win-3, the inactive cannabinoid, was compared with the active drug, Win-2, suggested otherwise. Win-3 did not produce any significant effects compared with Win-2. This stereo-selectivity indicates that Win-2 produced its effects through some receptor, possibly CB_1, CB_2 or an additional receptor yet to be identified. It is currently not known if chondrocytes express cannabinoid receptors and further studies are needed to establish this.

NO production in early phases of arthritis, in vivo, may lead to chondrocyte apoptosis [31]. Apoptotic programmed cell death of articular chondrocytes contributes to articular cartilage degradation [21]. Endogenously synthesized NO also reduces proteoglycan synthesis [32]. IL-1 via effects on the production of NO partially inhibits synthesis of type II collagen [21]. This NO-dependent inhibition may occur at the post-translational level, where collagen is subject to several processing stages including hydroxylation by prolyl hydroxylase, which may directly or indirectly be inhibited by NO [21]. NO also disassembles complexes formed at focal adhesion sites in response to binding of fibronectin to specific integrins, thereby affecting outside-in signalling in chondrocytes [33]. Thus, inhibition of NO production by cannabinoids may blunt the inhibition of the synthesis of proteoglycan and type II collagen synthesis, thus maintaining the differentiated status of the chondrocytes and preventing loss of chondrocytes through apoptosis. Cannabinoids may also be improving outside-in signalling in chondrocytes thus maintaining chondrocyte viability and the integrity of the cartilage extracellular matrix, ultimately preventing cartilage degradation. IL-1 also stimulates proteoglycan degradation by up-regulating aggrecanase, the proteolytic enzyme that cleaves the principal proteoglycan, aggrecan, in cartilage via the ceramide pathway [14]. It is speculated that cannabinoids may inhibit proteoglycan degradation by affecting this ceramide pathway.

In cytokine-stimulated chondrocytes, NO sustains nuclear translocation of NF-κB and thus keeps NF-κB-dependent transcription persistently switched on [34]. This may present a mechanism through which NO promotes cartilage degradation. Through this way NO may sustain or promote expression of proteinases responsible for the degradation of the extracellular matrix. This is supported by the report that selective inhibition of NF-κB blocks inflammatory bone destruction [35]. The increased release of sGAGs in the IL-1-stimulated cartilage explants seen in this study could be due to the action of NO produced by the stimulated chondrocytes. If this is the case, reduction of NO production would therefore reduce the release of sGAGs. It is possible that cannabinoids through their reduction of NO production by chondrocytes are reducing the release of sGAGs in the cartilage explants.

In conclusion, cannabinoids appear to have potential as cartilage protective agents by abrogating cartilage matrix degradation through their ability to inhibit NO production and proteoglycan degradation. Further studies are required to elucidate the mechanisms by which this occurs.

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