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**EFFECTS OF CANNABINOIDS ON IL-1 α -INDUCED
BOVINE CHONDROCYTE METABOLISM AND
CARTILAGE RESORPTION**

ESTERY CHIMWEMWE MBVUNDULA

A thesis submitted in partial fulfilment of the requirements of Sheffield
Hallam University for the degree of Doctor of Philosophy

August 2005

Sheffield Hallam University

ABSTRACT

Cannabinoids reported to have analgesic, anti-inflammatory and immunosuppressive effects were also shown to reduce joint damage in animal models of arthritis, suggesting that they have a potential as anti-arthritic agents. Therefore in this thesis, the effects of cannabinoids were studied to determine the mechanisms involved in their joint protective effects by making use of IL-1 α -induced bovine articular chondrocytes and nasal cartilage explants.

IL-1 α induction of the inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) pathways and effects of cannabinoids on these was studied using immunofluorescence, immunoblotting as well as ELISAs. Effects of cannabinoids on IL-1-induced activation of nuclear factor-kappa B (NF- κ B) as well as p38 mitogen activated protein kinase (MAPK) were determined by immunofluorescence and fast activated cell-based ELISA (FACE®). Cartilage explant cultures were stimulated with IL-1 α to resorb and effects of cannabinoids on the release of collagen (using hydroxyproline assay), cartilage oligomeric matrix protein (COMP) (using an ELISA) and proteoglycan (using dimethylmethylene blue assay) were studied as well. Expression of cannabinoid receptors in chondrocytes was also determined by immunoblotting and immunofluorescence.

Cannabinoids inhibited IL-1 α -induced iNOS, COX-2 expression, NF- κ B and p38 MAPK activation in chondrocytes. They also protected the explants from resorption stimulated by IL-1. Chondrocytes were also shown for the first time to express cannabinoid receptors. The results suggested that cannabinoids protected cartilage from cytokine-induced degradation, in part, by inhibiting NO and PGE₂ production and their pathways. These effects were possibly receptor mediated. Cannabinoids appear to have a significant potential as anti-arthritic therapeutics.

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ABBREVIATIONS

2-AG	2-Arachidonylglycerol
5-HPETE	5-hydroperoxyeicosatetraenoic acid
AA	Arachidonic acid
AC	Adenylate cyclase
ACEA	Arachidonyl-2'-chloroethylamide or <i>N</i> -(2-Chloroethyl)-5 <i>Z</i> ,8 <i>Z</i> ,11 <i>Z</i> ,14 <i>Z</i> -eicosatetraenamide
ADAM	A disintegrin and metalloprotease
ADAMTS	A disintegrin and metalloprotease with thrombospondin motifs
ADP	Adenosine diphosphate
AIDS	Acquired immune deficiency syndrome
AjA	Ajulemic acid
AM281	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl- <i>N</i> -4-morpholinyl-1 <i>H</i> -pyrazole-3-carboxamide
AM630	6-Iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1 <i>H</i> -indol-3-yl](4-methoxyphenyl)methanone
Anandamide	Arachidonylethanolamide or <i>N</i> -(2-Hydroxyethyl)-5 <i>Z</i> ,8 <i>Z</i> ,11 <i>Z</i> ,14 <i>Z</i> -eicosatetraenamide
AP-1	Activator protein-1
APC	Antigen presenting cells
BAC	Bovine articular cartilage
BH ₄	Tetrahydrobiopterin
BMA	British Medical Association
BNC	Bovine nasal cartilage
cAMP	Cyclic adenosine 3', 5'-monophosphate
CB ₁	Cannabinoid receptor-1
CB ₂	Cannabinoid receptor-2
CBD	Cannabidiol
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine-3', 5'-monophosphate
CIA	Collagen induced arthritis
CNS	Central nervous system
CO ₂	Carbon dioxide
COL1A1	Type I collagen gene
COMP	Cartilage oligomeric matrix protein
COX	Cyclooxygenase
cPGES	Cytosol/cytoplasmic PGE ₂ synthase
CS	Chondroitin sulphate
DMARDs	Disease modifying antirheumatic drugs
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dw	Dry weight
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FAAH	Fatty acid amide hydrolase
FAD	Flavin adenine dinucleotide
FBS	Foetal bovine serum

FLS	Fibroblast-like synoviocytes
FMN	Flavin mononucleotide
G1/G2/G3	Globular domain 1/2/3
GAG	Glycosaminoglycan
GC	Guanylyl cyclase or guanylate cyclase
G _{i/o} -protein	GTP binding protein with inhibitory alpha subunit
G-protein	GTP binding protein
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HU-210	6a <i>R</i>)- <i>trans</i> -3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6 <i>H</i> -dibenzo[b,d]pyran-9-methanol
IASP	International Association for the Study of Pain
ICE	Interleukin 1-Converting Enzyme
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IGD	Interglobular domain
IGF-1	Insulin-like growth factor-1
IGFBP	IGF-I binding protein
IGIF	IFN- γ -inducing factor
IL	Interleukin
IL-1R	IL-1 receptor
IL-1Ra	Interleukin-1 receptor antagonist
IL-4R	IL-4 receptor
iNOS (NOS II)	Inducible nitric oxide synthase
JWH-133	(6a <i>R</i> ,10a <i>R</i>)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6 <i>H</i> -dibenzo[b,d]pyran
kDa	Kilodaltons
KS	Keratan sulphate
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTA ₄	Leukotriene A ₄
LTB ₄	Leukotriene B ₄
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MK-886	1-[(4-Chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]- <i>a,a</i> -dimethyl-5-(1-methylethyl)-1 <i>H</i> -Indole-2-propanoic acid
MMP	Matrix metalloproteinases
MOPS	3-(<i>N</i> -morpholino) propanesulfonic acid
mPGES	Microsomal or membrane-associated PGE ₂ synthase
mRNA	Messenger ribonucleic acid
MT-MMP	Membrane-type MMPs
NaCl	Sodium chloride
NADA	<i>N</i> -Arachidonyldopamine or <i>N</i> -[2-(3,4-Dihydroxyphenyl)ethyl]-5 <i>Z</i> ,8 <i>Z</i> ,11 <i>Z</i> ,14 <i>Z</i> -eicosatetraenamide
NADP	Nicotinamide adenine dinucleotide phosphate

N-AG	N-Arachidonylglycine or N-(1-oxo-5Z,8Z,11Z,14Z-eicosatetraenyl)glycine
NaHCO ₃	Sodium bicarbonate
NaOH	Sodium hydroxide
NF-AT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-kappa B
NK	Natural Killer
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NS-398	N-[2-Cyclohexyloxy-4-nitrophenyl]methanesulfonamide
NSAIDs	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis
ODQ	H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
OSM	oncostatin M
PAGE	Polyacrylamide gel electrophoresis
PBM	Peripheral blood monocytes
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PEA	Palmitoylethanolamide
PGE ₂	Prostaglandin E ₂
PGG ₂	Prostaglandin G ₂
PGH ₂	Prostaglandin H ₂
PGS	Prostaglandin Synthase
PGs	Proteoglycans
PKA	Protein kinase A
PKC	Protein kinase C
PMN cells	Polymorphonuclear cells
RA	Rheumatoid arthritis
rER	Rough endoplasmic reticulum
ROM	Range of motion
SCW	Streptococcal cell wall
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SF	Synovial fluid
SFM	Synovial fluid monocytes
Std	Standard
TACE	TNF-alpha-converting enzyme
TGF-β	Transforming growth factor-beta
Th1/Th2	T cell helper-1/-2
THC	Δ ⁹ -tetrahydrocannabinol
TIMP	Tissue inhibitors of metalloproteinases
TNF-R55/-R75	55- /75-kDa TNF receptor
TNF-α	Tumour necrosis factor-alpha
TSP-1	Thrombospondin type 1
VEGF	Vascular endothelial growth factor
Win-55,212-2	R-(+)-[2,3-Dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate salt

Win-55,212-3

S(-)-[2,3-Dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl)-(1-naphthalenyl)methanone mesylate salt

ww

Wet weight

Δ^8 -/ Δ^9 -THC

Delta 8-/delta 9-Tetrahydrocannabinol

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CHAPTER 1

INTRODUCTION

General introduction

Arthritis, a term that literally means joint inflammation, is characterised by joint pain, swelling and stiffness followed by joint destruction and deformity (Auw Yang *et al.*, 2004; Blumenkrantz *et al.*, 2004). The most common forms of arthritis are osteoarthritis and rheumatoid arthritis, the most common disabling disease states in which articular cartilage metabolism is altered leading to cartilage destruction (Auw Yang *et al.*, 2004; van de Berg, 1998). There is no cure for these diseases although there are a number of drugs recommended for arthritis treatment such as the non-steroidal anti-inflammatory drugs and disease modifying anti-rheumatoid drugs (Arjmandi *et al.*, 2004; Carter, 1997). These drugs however are either slow-acting or may control either pain or inflammation, requiring combination of drugs, and have a number of side effects. The search for effective and less toxic anti-arthritic agents is still going on. Cannabinoids have potential as anti-arthritic agents. There is anecdotal evidence that they are effective in relieving symptoms of arthritis. Furthermore they have been shown to have analgesic, immunosuppressive and anti-inflammatory effects and to prevent joint damage in animal models of arthritis (Bidinger *et al.*, 2003; Burstein, 2000; Mafait *et al.*, 2000; Zurier *et al.*, 1998). It is therefore possible that cannabinoids have the potential to modify cartilage metabolism in arthritic conditions to prevent cartilage destruction. Studies on the effects of cannabinoids on cartilage metabolism form the core of this thesis.

1.1 Cartilage

Cartilage is a uniquely specialised connective tissue consisting of cells (chondrocytes) and extracellular matrix. There are 3 different types of cartilage characterised by composition of extracellular matrix namely: elastic cartilage, fibrocartilage and hyaline cartilage. Elastic cartilage is characterised by the presence of elastic fibres within the matrix, which increase elasticity in tissues such as the external ear and trachea (Saw and Mironowicz, 1991). Fibrocartilage is found in intervertebral discs, the pubic symphysis, in menisci of joints, and often occurs where tendon and ligament are joined to bones.

This tissue consists of thick, interlaced collagenous fibres, collagen type I is the most abundant collagen in this cartilage. Fibrocartilage provides good resistance to shear and compression forces (Saw and Mironowicz, 1991). Hyaline cartilage is found lining articular surfaces, and in the nasal septum, tracheal rings, costal cartilages, and the epiphyseal cartilage of growing bone. Articular hyaline cartilage consists of a glassy and homogenous matrix with more collagen than other types of hyaline cartilage (Saw and Mironowicz, 1991).

1.1.1 Articular cartilage

Articular cartilage is a very important tissue of the diarthrodial joints (also known as synovial joints) (Fig. 1.1). These joints have a wide range of movement. The joint cavity in these joints is fairly large with the adjacent bones widely separated and is filled with synovial fluid which lubricates the joints (Stevens and Lowe, 2000). Cavity margins are defined partly by articular cartilage covering the ends of the adjacent bones, and partly by a tough fibrous capsule (Stevens and Lowe, 2000; Woolf, 1998). The inner surface of the capsule is lined by well vascularised connective tissue, composed partly of fibrous and partly of adipose tissue. This is lined by synovial membrane (synovium), which is a layer or two of type A cells (belonging to the macrophage family, producing hyaluronic acid) and type B cells (fibroblastic but also producing non-collagenous proteins). There is no basement membrane beneath the synovium, hence transfer of water, solutes and proteins between the blood and fluid within the cavity is facilitated (Woolf, 1998).

Articular cartilage has unique material properties that enable it to perform its physiological and mechanical functions over a lifetime and under a wide range of loading conditions (Muir, 1980; Roughley, 2001). Articular cartilage is a tissue in which cells (chondrocytes) are sparsely distributed in rigid extracellular matrix (Muir, 1980). The articular cartilage is avascular, aneural and alymphatic relying on the process of diffusion to provide nutrients for the

chondrocytes (Roughley, 2001). Access to nutrients and elimination of waste products occur via diffusion through the extracellular matrix (Roughley, 2001).

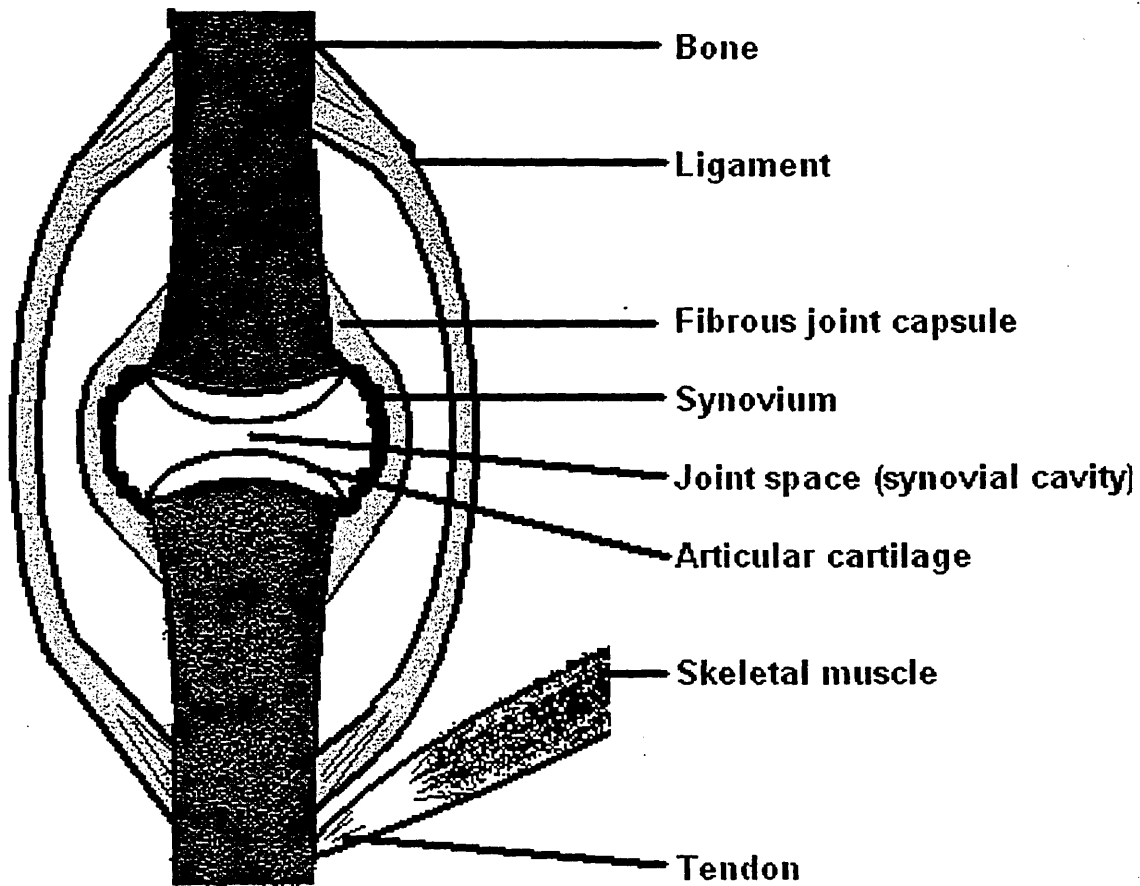


Figure 1.1: Diagram of a synovial joint showing the features that make up the joint and help it to articulate and make a wide range of movements.

1.1.2 Articular cartilage composition

Articular cartilage is primarily made up of water, collagen, proteoglycans, other non-collagenous proteins and chondrocytes.

1.1.2.1 Water

Articular cartilage is a highly hydrated material. Water distribution varies; making up 65% of wet weight at the deep zone and 80% at the joint surface (Muir, 1980; Saw and Mironowicz, 1991). Weight bearing capacity is made

possible through regional changes in water content which allow deformation of the cartilage surface in response to stress (Saw and Mironowicz, 1991). It is precisely the presence of water in conjunction with the hydrophilic proteoglycans and presence of collagen that makes cartilage tough and resilient (Muir, 1980). A random macromolecular mesh (represented here by proteoglycans), when placed within a fibrous network (such as collagen) such that the macromolecule cannot move, impedes the flow of interstitial water within the tissue when an external force is applied. Fluid pressure within cartilage rises immediately a load is applied, but cartilage deforms only gradually as the water is driven out from the loaded areas. Thus the compressive stiffness of cartilage over short time intervals is directly correlated with the proteoglycan content measured as glycosaminoglycans (GAGs) (Muir, 1980).

Water acts as a medium through which chondrocytes have access to nutrients and eliminate wastes and it also provides lubrication of cartilage (Saw and Mironowicz, 1991). Water content decreases with normal ageing (Saw and Mironowicz, 1991) whilst water content of osteoarthritic cartilage increases, particularly in coarse, fibrillated areas where the GAG content is markedly depleted (Muir, 1980). Water distribution in the tissue is also altered, it is highest near the mid-zone rather than toward the joint surface as in normal cartilage (Muir, 1980). It was reported also that unlike normal cartilage, osteoarthritic cartilage swells when placed in 0.15 M NaCl and it has been suggested that osteoarthritic cartilage imbibes water and swells in physiological saline because the collagen network that opposes swelling pressure of proteoglycan has been damaged (Muir, 1980).

1.1.2.2 Collagen

Collagen forms the framework that provides the tensile strength and stiffness in articular cartilage (Sugimoto *et al.*, 2004). It accounts for about 50% dry weight (dw) of the human adult articular cartilage, however articular cartilage is not uniform in composition throughout its depth (section 1.1.3). The collagen content is higher in the superficial layers. Proteoglycan content

varies inversely with the collagen content since it is highest in the deeper layers of the cartilage tissue (Muir, 1980).

Articular cartilage contains a number of genetically distinct types of collagen (Eyre, 1991). Type II collagen is the most abundant collagen in mammals, however collagens III, VI, IX, X, XI, XII and XIV all contribute to the mature matrix (Eyre, 2002). Collagen types II, IX, X, and XI are referred to as "cartilage specific". Collagen types II, IX, and XI are organized into copolymeric fibrils, where type II constitutes the bulk of the fibril, type XI regulates fibril size, and type IX facilitates fibril interaction with proteoglycan macromolecules (Cremer *et al.*, 1998). Stromelysin, a matrix metalloproteinase, was recently shown to degrade type IX collagen. This action may cause the collagen network swelling seen in articular cartilage in early experimental osteoarthritis (OA) (Eyre, 1991). Collagen type X is restricted to the thin layer of calcified cartilage that interfaces articular cartilage with bone (Eyre, 2002), a zone that exhibits active remodeling in joints with OA (Eyre, 1991).

Chondrocytes are surrounded by a pericellular matrix and enclosed within a pericellular capsule and it has been suggested that the chondrocyte, its pericellular matrix, and capsule together constitute the chondron. Type VI collagen is preferentially localised in the chondron (Poole *et al.*, 1992) and may help the chondrocytes to attach to the macromolecular framework of the matrix (Buckwalter *et al.*, 1998).

Synthesis of collagen takes place in stages at various intracellular and extracellular sites. Intracellularly, collagen mRNA is translated into polypeptide chains that are released into the cisternae of the rough endoplasmic reticulum (rER) where the signal peptide is cleaved. Lysine and proline residues are hydroxylated and hydroxylysine residues are glycosylated. N-linked sugars are added to the terminal portion of the polypeptide (Saw and Mironowicz, 1991). The collagen molecule consists of an uninterrupted triple helix of about 300 nm in length and 1.5 nm in diameter. This is formed from three component polypeptide chains of repeating

structure G-X-Y, in which G is Glycine; X and Y are frequently the imino acids proline and hydroxyproline (Holmes *et al.*, 2001). Thus procollagen is formed through intrachain and interchain disulfide bonds that stabilise the polypeptides and determine the shape of the molecule. Procollagen is then packed into secretory granules, which move along microtubules to be released into the extracellular matrix (Saw and Mironowicz, 1991). Extracellularly, uncoiled terminal ends of procollagen are cleaved to form tropocollagen. Tropocollagen molecules aggregate and lysine and hydroxylysine residues are crosslinked to form collagen fibrils, which also aggregate to form collagen fibres (Saw and Mironowicz, 1991) (see Fig. 1.2).

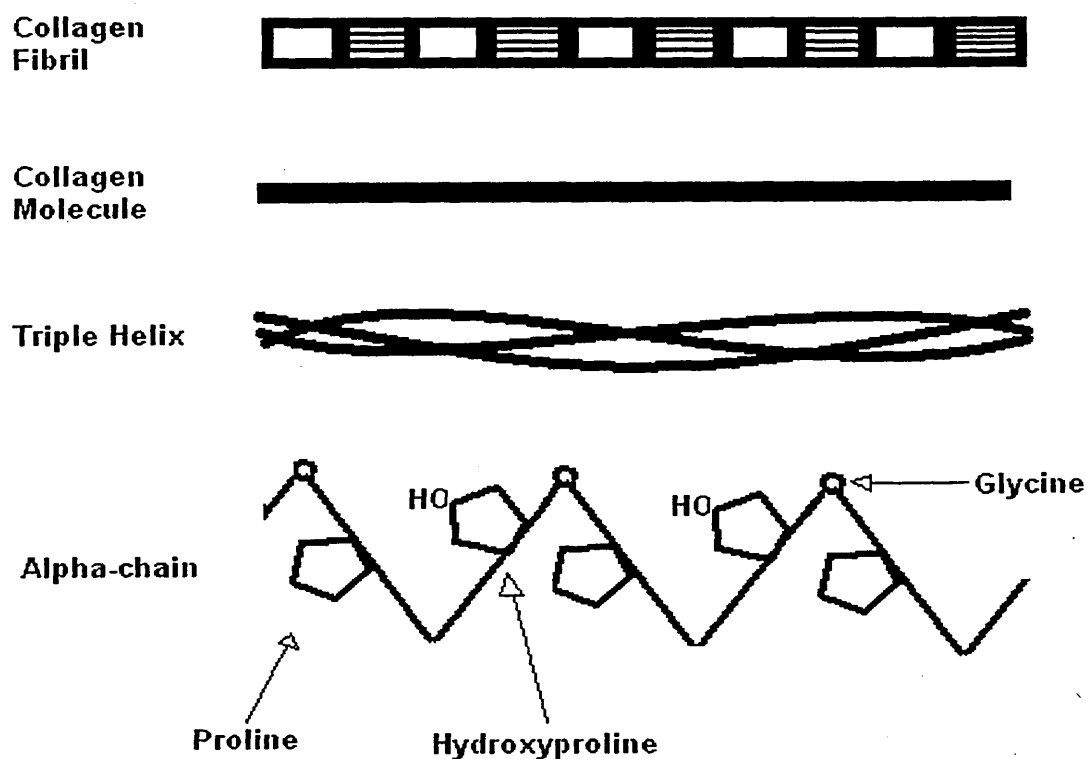


Figure 1.2: Diagram showing stages of formation and arrangement of collagen. Modified from Saw and Mironowicz (1991).

1.1.2.3 Proteoglycans and other non-collagenous proteins

Collagen molecules and proteoglycans interweave to form cartilage matrix. Proteoglycans, due to their polyanionic charge provided by the sulphated

GAG chains, attract water into the cartilage matrix, thus creating a high osmotic pressure resisted by and constrained only by the framework of collagen fibres (Little *et al.*, 2002). During joint loading and articulation, the proteoglycans dissipate the compressive forces and reversibly deform by releasing water to the synovial fluid, and upon relaxation they pull water in (Flannery *et al.*, 1999; Sugimoto *et al.*, 2004). This model of osmotic pressure and collagen fibres, thus, renders the cartilage resistant to major irreversible deformation during load-bearing, and allows cartilage resiliency upon removal of load.

Aggrecan

The large aggregating proteoglycan, aggrecan, which is responsible for the osmotic swelling capability, and thus the elasticity of the cartilage matrix, is the most abundant non-collagenous protein in cartilage (Sugimoto *et al.*, 2004).

Aggrecan consists of an extended protein core, which can be subdivided into several well-defined functional regions: the N-terminal globular G1, G2 domains and an interglobular domain (IGD) between the G1 and G2 domains, the keratan sulfate and chondroitin sulfate attachment region, and the C-terminal globular G3 domain (see Fig. 1.3) (Sugimoto *et al.*, 2004).

The G1 domain non-covalently links with hyaluronic acid and a protein called link protein stabilises the association of the two molecules (Cawston, 1998). The attachment of approximately a hundred aggrecan monomers to a hyaluronic acid molecule form the multimolecular proteoglycan aggregates (Caterson *et al.*, 2000).

The IGD domain, the short polypeptide region between G1 and G2, is highly conserved between mammalian species (Flannery *et al.*, 1999) and it is the area on the core protein that is most prone to attack by proteinases.

The three major GAGs found in cartilage in order of abundance are chondroitin sulfate, keratan sulfate and hyaluronic acid. Numerous chains of chondroitin sulfate and keratan sulfate covalently linked to a core protein constitute the aggrecan monomer. Keratan sulphate (KS) attachment domain follows the G2 domain on the core protein of aggrecan. In turn, this is followed by the chondroitin sulphate (CS) attachment region (Fig. 1.3). Over 100 CS and KS chains may be present in the GAG attachment domains (Roughley, 2001). The C-terminal globular domain, G3, possibly interacts with other components of the extracellular matrix, or may be involved in the intracellular trafficking during aggrecan synthesis (Roughley, 2001).

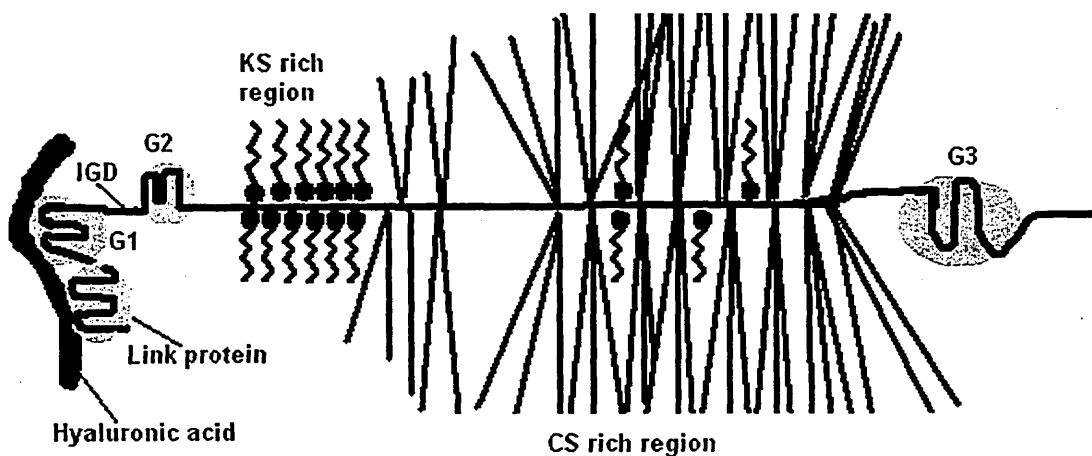


Figure 1.3: Diagram showing an aggrecan molecule linked to hyaluronic acid and stabilised by link protein. Modified from Porter *et al.* (2005).

Small leucine-rich repeat proteoglycans

The small proteoglycans (PGs) of cartilage matrix represent a small fraction of the total mass of PGs, but with a small size they can be present in equivalent moles to the large PGs. Three types of PGs with a wide skeletal and extraskeletal distribution, biglycan (PGI), decorin (PGII) and fibromodulin have distinct but homologous core proteins containing leucine-rich sequences. Carbohydrate substituents (one or two chondroitin sulphate or dermatan sulfate chains for decorin and biglycan respectively, chains of keratan sulfate for fibromodulin and oligosaccharides) present variations from tissue to tissue

and with age and other factors. Decorin and fibromodulin appear to interact with collagen and to participate in the regulation of collagen matrices. In vitro experiments indicate a role for small PGs in adhesion, multiplication, differentiation, and migration of cells (Stanescu, 1990).

Cartilage oligomeric matrix protein (COMP)

The extracellular matrix of cartilage contains numerous proteins that are neither collagens nor proteoglycans and most of these are thought to play structural roles in the matrix. COMP is probably the most studied of these proteins (Roughley, 2001). COMP is a 524 kDa glycoprotein component of the matrix. It is composed of 5 100-120 kDa subunits and is secreted by chondrocytes from various types of cartilage and chondrosarcoma cells (Newton *et al.*, 1994). It is the fifth member of the thrombospondin (TSP) gene family with a bouquet-like molecular structure (Fig. 1.4) (Posey *et al.*, 2004).

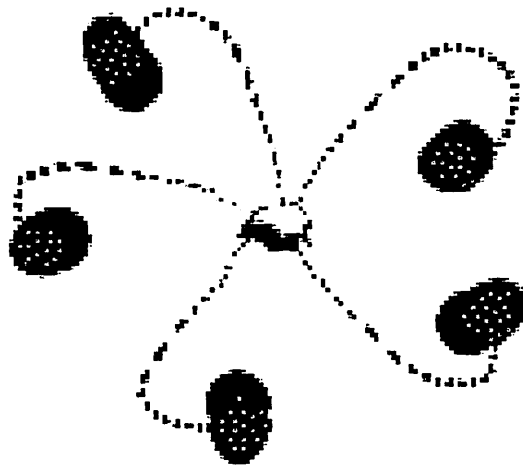


Figure 1.4: A diagram of COMP pentamer showing the bouquet arrangement with the globular domains at the ends of each monomeric unit. Modified from Posey *et al.* (2004)

Functional studies have shown that COMP binds types II and IX collagens but the role of COMP in the extracellular matrix remains to be defined (Posey *et al.*, 2004). COMP also binds to fibronectin. Confocal microscopy

demonstrated punctate binding on the cell surface, which is indicative of possible binding to cell surface receptors. It has been found that chondrocytes bind to fibronectin by an integrin receptor and that this complex is in turn anchored to the collagen matrix by a complex network mediated by COMP (DiCesare *et al.*, 2002).

1.1.2.4 Chondrocytes

Structural integrity and cartilage function are maintained by the resident, sparsely distributed chondrocytes that rely on the diffusion of oxygen and nutrients from the synovial fluid or capillaries in the subchondral bone (Bigg and Rowan, 2001). Chondroprogenitor cells which are derived from mesenchymal cells differentiate into chondrocytes enclosed within small cavities called lacunae, often in groups of 2, 4, or 6 cells as a result of mitosis and restricted cellular movement (Saw and Mironowicz, 1991).

Chondrocytes are important in the control of matrix turnover through production of collagen, proteoglycans and enzymes for cartilage metabolism. The available evidence indicates that normal matrix turnover depends on the ability of chondrocytes to detect alterations in the matrix composition and organization, including the presence of degraded molecules, and to respond by synthesizing appropriate types and amounts of new molecules (Bulkwalter and Mankin, 1998).

1.1.3 Articular cartilage zonal arrangement

The articular cartilage contains different zones with respect to depth from the articular surface and has a regional organization around the chondrocytes. Its composition varies regionally and zonally in its collagen and proteoglycan contents and those of other matrix molecules (Poole *et al.*, 2001). Beginning with the articulating surface, mature cartilage can be separated into the superficial, middle, deep, and calcified zones (Fig. 1.5). The first 10–20% of the full-thickness articular cartilage contains densely packed collagen II fibrils and flattened, discoid or elongated flattened cells, oriented parallel to surface

in the direction of shear stress and is classified as the superficial zone (Saw and Mironowicz, 1991; Darling *et al.*, 2004). The middle zone (40–60% of full thickness) contains round cells arranged singly or in pairs and collagen fibres that are thick and obliquely or randomly arranged. It serves as a transition between the superficial and deep zones (Darling *et al.*, 2004). Columns of ellipsoid cells distributed between radially oriented collagen fibres characterize the deep zone. The collagen fibrils extend into the calcified zone to reinforce the bond between cartilage and bone. The deep zone is separated from the calcified zone by a distinct tidemark, an undulating barrier tangential to the surface, which represents a plane of weakness, which is often considered the boundary between cartilage and bone. The calcified zone contains cells trapped within a calcified matrix and it contains hydroxyapatite crystals that anchor the cartilage to the subchondral bone (Darling *et al.*, 2004; Saw and Mironowicz, 1991).

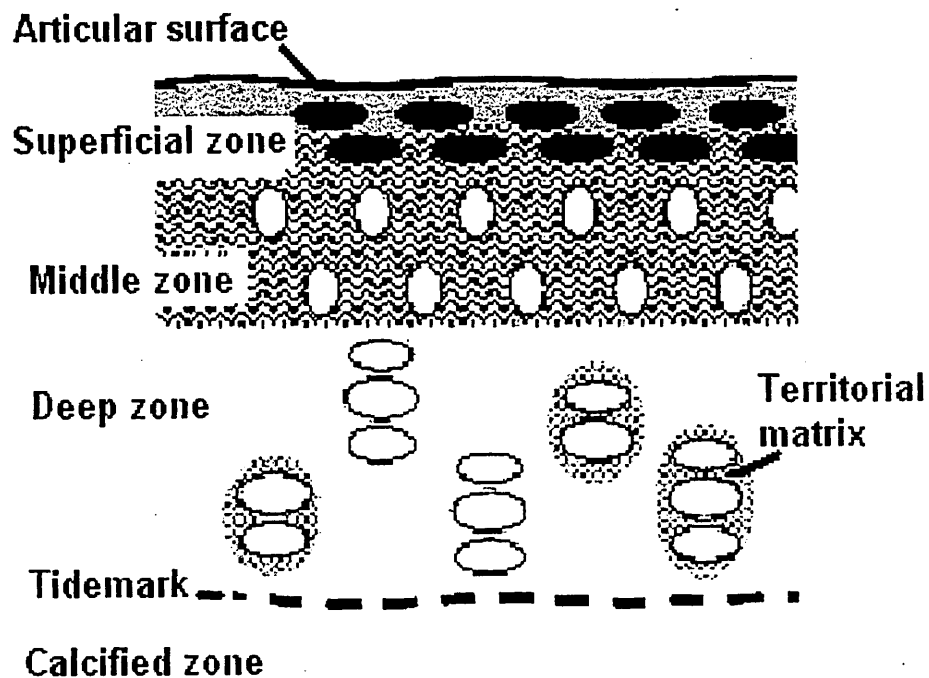


Figure 1.5: Diagram showing different zonal layers of articular cartilage. Adapted from Darling *et al.* (2004).

Through the depth of cartilage, water content falls linearly, from approximately 84% wet weight (ww) to 40–60% ww. The collagen content falls from 86% dw in the superficial zone to 67% dw in the deep zone. The proteoglycan content increases from around 15% dw in the superficial zone to a peak of 25% dw in the middle zone, then falls to 20% in the deep zone Darling *et al.* (2004).

1.2 Cartilage Metabolism

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 (Steinmeyer and Daufeldt, 1997). Under physiologic conditions, chondrocytes show steady-state equilibrium between anabolic and catabolic activities that maintains the structural and functional integrity of the cartilage extracellular matrix (Goldring *et al.*, 2004). Any change in this steady state will rapidly affect the healthy function of the cartilage (Cawston, 1998). Active proteinases such as aggrecanases and matrix metalloproteinases (MMPs) are involved in cartilage resorption by degrading major components of cartilage extracellular matrix, collagen and proteoglycan, mostly type II collagen and aggrecan, respectively (Cawston, 1998). The MMPs are secreted from the cells in a latent form (zymogens), requiring activation extracellularly. They and aggrecanases are inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Steinmeyer and Daufeldt, 1997). In arthritic conditions such as osteoarthritis and rheumatoid arthritis there is a disparity between the proteinases and TIMPs that accounts at least in part for the observed cartilage destruction (Steinmeyer and Daufeldt, 1997; Cawston, 1998). Proteoglycan breakdown is an early event in cartilage resorption process, while substantial degradation of collagen occurs later and may represent the point of irreversible cartilage damage (Little *et al.*, 2002).

1.2.1 Cytokines in Cartilage Metabolism

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 (Arner *et al.*, 1998; Flannery *et al.*, 2000; Lubberts, 2003), whilst protective effects against cartilage breakdown were shown by IL-4, IL-10, IL-13, transforming growth factor- β 1 (TGF- β 1) and insulin-like growth factor-1 (IGF-1) (Cawston *et al.*, 1996; Lubberts *et al.*, 1998; Koshy *et al.*, 2002).

1.2.1.1 Catabolic Cytokines

Key proinflammatory cytokines, IL-1 and TNF- α

IL-1 was originally known as lymphocyte-activating factor (Saklatvala *et al.*, 1985). Around the same time, a soluble factor, termed catabolin, released from living synovial and other connective tissues was reported to be capable of stimulating chondrocytes to degrade totally both proteoglycan and collagen (Dingle, 1981). The synovial catabolin was later believed to be a form of IL-1 (Yodlowski *et al.*, 1990) and two immunologically distinct forms of IL-1 were purified and found to have identical molecular weight, demonstrate the same range of biological activity, but differ in isoelectric point. These isoforms were identified as IL-1 α and IL-1 β (Saklatvala *et al.*, 1985). IL-1 is the prototype of the pro-inflammatory cytokines in that it induces the expression of a variety of genes and the synthesis of several proteins that, in turn, induce acute and chronic inflammatory changes. IL-1 is also the prototypic cytokine that brings about increases in a variety of defense mechanisms, particularly immunologic and haematologic responses (Dinarello, 1991). The IL-1 family includes two agonist proteins, IL-1 α and IL-1 β , which are able to trigger cell activation upon interaction with specific receptors on the membrane of target cells (Boraschi *et al.*, 1996). Both members are first synthesized as 31 kDa precursor proteins, proIL-1 α and proIL-1 β respectively (Boraschi *et al.*, 1996). The pro-IL-1 α and the enzymatically cleaved mature 17 kDa IL-1 α are both biologically active. Approximately 10-15% of the IL-1 α is myristoylated. It is believed that this form is transported to the cell surface where it is attached to the membrane and is called membrane IL-1 (Dinarello, 1994; Boraschi *et al.*, 1996). In contrast, proIL-1 β is relatively inactive and requires cleavage to a 17 kDa peptide for optimal biological activity (Dinarello, 1994). An intracellular

cysteine protease known as Interleukin 1-Converting Enzyme (ICE also known as caspase-1) is highly specific for cleaving proIL-1 β but does not cleave the IL-1 α precursor. Other enzymes have been found that do cleave the IL-1 precursors but these seem less specific than ICE (Boraschi *et al.*, 1996). IL-1 proteins lack a signal peptide and thus are not secreted through classical pathways and are unglycosylated proteins (Boraschi *et al.*, 1996). The third member of the IL-1 family is IL-1Ra, a pure receptor antagonist, which is a glycosylated secretory protein of about 23 kDa (Boraschi *et al.*, 1996; Dinarello, 1994). IL-1Ra is also first produced as a precursor with a leader sequence (peptide signal), which is cleaved to produce the mature form therefore IL-1Ra is secreted like most proteins (Boraschi *et al.*, 1996). Although IL-1Ra is secreted (sIL-1Ra), another form of the IL-1Ra gene is retained inside cells: intracellular (ic) IL-1Ra. icIL-1Ra results from alternate mRNA splicing of the IL-1Ra transcript replacing the first exon coding for the signal peptide. However, both forms of IL-1Ra are functionally indistinguishable. IL-1Ra functions primarily to block IL-1 receptors and thus acts as a natural inhibitor of IL-1 activity (Dinarello, 1994).

Activation of target cells by IL-1 depends on the interaction with specific receptors on the cell membrane. Two types of IL-1 receptors (IL-1R) have been identified, IL-1R_I and IL-1R_{II}. The second type of IL-1R, IL-1R_{II}, shares many structural characteristics of IL-1R_I. The main difference between IL-1R_{II} and IL-1R_I lies in the intracellular domain, which is extremely short for IL-1R_{II} and is apparently unable to initiate signal transduction. Thus, the functional role of IL-1R_{II} apparently is that of binding and sequestering IL-1, thus avoiding its interaction with IL-1R_I and consequent cell triggering. IL-1R_{II} can therefore act as another natural inhibitor of IL-1 activity, complementing the function of IL-1Ra (Boraschi *et al.*, 1996).

TNF- α is a proinflammatory cytokine that contributes to a variety of disease states and is produced mainly by monocytes and macrophages (Shankar and Handa, 2004). The protein exists as a membrane-bound precursor of relative molecular mass of 26 kDa which can be processed by a TNF-alpha-converting enzyme (TACE also known as ADAM17), to generate secreted 17

kDa mature TNF- α (Moss *et al.*, 1997). TNF α binds to its receptors on a variety of target cells, transducing a signalling cascade in those cells. There are two TNF-alpha receptors; the 55 and 75 kDa TNF- α receptors (TNF-R55 and TNF-R75, respectively) (Gallea-Robache *et al.*, 1997) also referred to as p55 and p75 TNF receptors (Webb *et al.*, 1997). Expression of p55 TNF- α receptor has been localised in cells at sites of focal loss of cartilage proteoglycan in OA patients (Webb, *et al.*, 1997) providing evidence for role of TNF α in pathogenesis of human OA. TNF- α triggers production of other cytokines, induces endothelial adhesion molecules, stimulates collagenase and stromelysin production, and stimulates osteoclast differentiation thus its blockade has a more global effect on inflammation than the blockade of other cytokines (Shankar and Handa, 2004).

A number of studies showed that effects of TNF α were similar to IL-1 or synergistic with it and thus suggested a role for the cytokines in cartilage destruction (Goldring, 2000). IL-1 is one of the key mediators by which the chondrocytes and synoviocytes enhance their protease production (Steinmeyer and Daufeldt, 1997) and inflammatory factors. 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 alone or in combination with TNF α (Amin and Abramson, 1998; Goldring, 2000). IL-1 alone or in combination with TNF α has also been shown to be capable of stimulating chondrocytes to express cyclooxygenase-2 (COX-2) which results in production and secretion of prostaglandin E₂ by the cells (Hardy *et al.*, 2002; Martel-Pelletier *et al.*, 2003). These pro-inflammatory mediators have been implicated in the pathogenesis of osteoarthritis (Goggs *et al.*, 2003; Mendes *et al.*, 2002; Studer *et al.*, 1999). They have different effects in articular cells that may lead to cartilage resorption. These effects include inhibition of matrix synthesis, upregulation of MMP production and induction of chondrocyte apoptosis (Goggs *et al.*, 2003; Henrotin *et al.*, 2003; Studer *et al.*, 2003). Apoptosis induced by PGE₂ in chondrocytes is linked with the cAMP-dependent pathway (Miwa *et al.*, 2000). Both IL-1 and TNF α are found at enhanced levels in synovial fluid from inflamed joints and are thought to play important roles in the destruction that ensues (Manicourt *et*

al., 2000). Through use of animal models of arthritis and cytokine-specific neutralising antibodies as well as cytokine specific knockout conditions, it was found that TNF α is sufficient to drive the early aspect of joint inflammation as well as the acute cellular infiltrate in the synovial tissue, while IL-1 has a pivotal role in sustaining both inflammation and erosive cartilage damage (van den Berg *et al.*, 1999). Persistent joint inflammation and erosive joint damage are the characteristics of chronic arthritis thus both cytokines need to be targeted to achieve optimal treatment in arthritis (van den Berg *et al.*, 1999).

Other proinflammatory cytokines

IL-6

IL-6 is one of the regulatory cytokines that influences IL-1- and TNF α -induced catabolic processes (Goldring, 2000). IL-6 is involved in diverse biological processes such as final maturation of B cells into plasma cells, T cell activation, induction of the acute-phase response, stimulation of growth and differentiation of haematopoietic precursor cells and proliferation of synovial fibroblasts (Shankar and Handa, 2004). IL-1 and TNF α can stimulate its synthesis in chondrocytes (Goldring, 2000). IL-6 alone is not capable of stimulating cartilage degradation directly. Recently it has been reported that soluble IL-6 receptor (sIL-6R α) is required for full response to IL-6 in chondrocytes *in vitro* and was also found to potentiate the inhibitory effects of IL-6 on proteoglycan synthesis, but this effect was moderate compared with that of IL-1 (Goldring, 2000). IL-6 and its soluble receptor also augment aggrecanase-mediated proteoglycan catabolism in articular cartilage (Flannery *et al.*, 2000).

IL-17

IL-17 is one of the potent inducers of catabolic responses in chondrocytes. It increases the expression of IL-1 β , IL-6, stromelysin, iNOS and COX-2 in human articular chondrocytes (Goldring, 2000). IL-17 is produced by activated T lymphocytes, predominantly of CD4+ sub-type, in RA synovial

fluid and tissue (Goldring 2000). Interleukin 17 induces cartilage collagen breakdown in cartilage and shows synergistic effects in combination with other proinflammatory cytokines (Koshy et al., 2002) and IL-17 has a role in the pathogenesis of arthritis (Lubberts, 2003).

IL-18

IL-18 is a cytokine that was originally identified as Interferon-gamma (IFN- γ)-inducing factor (IGIF) (Ghayur *et al.*, 1997). IL-18 has structural similarities with the IL-1 family of proteins. IL-18 is also synthesized as a biologically inactive precursor and cleaved by the ICE. Macrophages and other cells produce it. The human IL-18R was purified and its sequence was found to be identical to the previously known IL-1R-related protein (Olee *et al.*, 1999). IL-18 augments natural killer (NK) activity in spleen cells and may be involved in the development of Th1 cells and also in mechanisms of tissue injury in inflammatory reactions (Okamura *et al.*, 1995). IL-18 stimulates the expression of several genes and their proteins in normal human articular chondrocytes including iNOS, COX-2, IL-6, and stromelysin and treatment of normal human articular cartilage with IL-18 increased proteoglycan degradation (Olee *et al.*, 1999). This suggests that IL-18 regulates chondrocyte responses and contributes to cartilage pathophysiology. Synovial tissue IL-18 expression was also shown to correlate with disease activity in inflammatory arthritis (Rooney *et al.*, 2004). After treatment, tissue levels changed in parallel with changes in serum IL-18 and with changes in the acute phase response. These observations support a role for IL-18 in the pathophysiology of inflammatory arthritis (Rooney *et al.*, 2004).

Oncostatin M

Oncostatin M (OSM), a T cell or monocyte cytokine, closely related to IL-6, stimulates proteoglycan release and inhibits its synthesis in cartilage explants. Elevated levels of OSM were detected in synovial fluid from arthritic patients (Hui *et al.*, 1997). When in combination with IL-1 α , OSM promoted an enhanced release of proteoglycan and collagen from cartilage explants. This

was accompanied by an enhanced upregulation of active and pro-collagenases and a reduction in TIMP-1 (Hui *et al.*, 2000). Unlike IL-6, OSM stimulated aggrecan catabolism and when combined with IL-1 enhanced release of collagen in nasal cartilage explants (Manicourt *et al.*, 2000).

1.2.1.2 Immunomodulatory and anti-inflammatory cytokines

IL-4

IL-4 is a 20 kDa glycoprotein that has a broad spectrum of biological activities including growth and differentiation of T cells, B cells, mast cells, haematopoietic cells and non-haematopoietic cells, such that it is referred to as the prototypic immunoregulatory cytokine (Brown and Hural, 1997; Noma, 1992). It is produced only by a subset of activated hematopoietic cells, including T cells, mast cells and basophils and has an important role in regulating antibody production, haematopoiesis and inflammation, and the development of effector T-cell responses (Brown and Hural, 1997). The biological effects of IL-4 are induced by binding to specific membrane IL-4 receptors (IL-4R) on target cells (Noma, 1992). IL-4 blocks the release of collagen fragments from bovine nasal cartilage treated with cytokines (Cawston *et al.*, 1996), suggesting its therapeutic potential in arthritis. IL-4 also enhances the effects of IL-10 in reducing joint inflammation and cartilage destruction in murine streptococcal cell wall (SCW) arthritis (Lubberts *et al.*, 1998) producing more therapeutic benefits.

IL-10

IL-10, an immunoregulatory cytokine produced by T cells and monocytes, inhibits the expression of inflammatory and hemopoietic cytokines as well as its own expression (Daftarian *et al.*, 1996). IL-10 inhibits the antigen-presenting capacity of synovial fluid (SF) macrophages, importantly, IL-10 is able to downregulate the antigen presenting function of SF macrophages even when they are efficiently activated, which further emphasizes the anti-inflammatory potential of IL-10 in RA (Mottonen *et al.*, 1998). IL-10 is

spontaneously produced in RA and OA and is an important immunoregulatory component in the cytokine network of RA (Katsikis *et al.*, 1994) and has been shown to play a role in RA as an anti-inflammatory cytokine (Isomaki *et al.*, 1996). Neutralization of endogenously produced IL-10 in RA synovial membrane cultures resulted in a two- to threefold increase in the protein levels of proinflammatory cytokines (IL-1 β and TNF α) (Katsikis *et al.*, 1994). IL-10 also enhances IL-4 suppression of inflammatory cytokine production by rheumatoid synovial cells (Sugiyama *et al.*, 1995).

IL-13

Interleukin 13 (IL-13) is a recently described cytokine secreted by activated T cells (Zurawski and de Vries, 1994). IL-13 has profound effects on monocytes *in vitro*, acting not simply to deactivate them but rather in a complex manner to change monocytic morphology, phenotype, function, and cytokine production (Woods *et al.*, 2000). IL-13 shares biological activities with IL-4, their genes are closely linked and there is sequence homology between IL-13 and IL-4 proteins. Although the cloned IL-4 receptor protein (IL-4R) does not bind IL-13, it appears that the functional IL-4R and IL-13R share a common subunit that is important for signal transduction (Zurawski and de Vries, 1994). IL-13 is potentially useful in the therapeutic treatment of OA, as it could regulate the major pathological process of this disease by reducing the production of proinflammatory cytokines (IL-1 β and TNF α) and metalloproteases (e.g. stromelysin-1), and favoring the production of IL-1Ra (Jovanovic *et al.*, 1998). Increased expression of IL-13 via gene therapy decreased RA-associated inflammation by reducing secretion of proinflammatory cytokines and PGE₂ in RA synovial tissue explants (Woods *et al.*, 2000). In animal models of collagen induced arthritis (CIA), vector cells engineered to secrete interleukin-13 attenuated CIA in mice and this effect coincided with a decreased TNF- α mRNA expression in the spleen of treated animals (Bessis *et al.*, 1996), suggesting therapeutic potential of IL-13 in the treatment of autoimmune diseases such as RA.

In one study, effects of IL-4, IL-10, and IL-13 were compared in the interruption of two major inflammatory pathways in RA (i.e. overexpression of proinflammatory cytokines and cytokine-mediated fibroblast growth). All the cytokines were able to significantly inhibit the production of proinflammatory cytokines by freshly isolated RA synovial tissue cells; however IL-10 was the most potent. The IL-1 receptor antagonist was enhanced by IL-4 and IL-13, but only slightly enhanced by IL-10. Addition of anti-IL-10 neutralizing antibody to RA synovial tissue cells resulted in a substantial increase in IL-1 β and TNF- α levels, whereas neither anti-IL-4 nor anti-IL-13 antibody had a significant effect. IL-1 β -stimulated proliferation of RA synovial fibroblast cell lines was inhibited by IL-4 and IL-13, but not by IL-10; IL-4 was over tenfold more effective than IL-13. The results indicate that cytokines, IL-4, IL-10, and IL-13, have the therapeutic potential to regulate the disease activity mediated by proinflammatory cytokines in RA, but each cytokine may have different potencies (Morita *et al.*, 2001).

1.2.1.3 Growth factors in cartilage

Transforming growth factor-beta (TGF- β s)

The TGF family includes a number of polypeptides involved in the regulation of cell growth and differentiation and in wound healing. The TGF- β , first identified by its ability to stimulate normal cells to grow and differentiate in soft agar, is found throughout the body and binds to at least three membrane receptors common to most cells. Three isoforms, TGF- β 1, TGF- β 2, and TGF- β 3, have been isolated from cartilage in mammals (Holland and Mikos, 2003).

TGF- β 1 has been shown to promote the differentiation of stem cells to osteoblasts, osteoclasts, and chondrocytes. In articular cartilage, TGF- β 1 promotes protein synthesis and cell proliferation and inhibits the actions of MMPs (Holland and Mikos, 2003). In addition to these protective effects on articular cartilage, it is also known to promote chemotaxis and activation of inflammatory cells and, at high concentrations, has been implicated in fibrosis

and osteophyte formation in articular cartilage defects (Holland and Mikos, 2003).

IGFs

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), which exhibit close homologies to insulin, have been isolated from a number of tissues, including articular cartilage. These growth factors were initially discovered as sulfation factors or somatomedins, which promote sulfate incorporation into proteoglycans. In articular cartilage, these growth factors act primarily in an anabolic fashion to increase proteoglycan and type II collagen synthesis. IGF-1 also appears to influence cellular differentiation and specific integrin expression (Holland and Mikos, 2003). Although IGF-1 is nearly ubiquitous, its bioavailability in cartilage is controlled by IGF-1 binding proteins (IGFBPs) secreted by chondrocytes. IGFBPs are part of a complex system, termed the IGF-1 axis that tightly regulates IGF-1 activities. For the most part, IGFBPs block IGF-1 activity by sequestering IGF-1 from its cell surface receptor controlling its bioavailability in human articular cartilage (Martin *et al.*, 2000). The damage-induced proteinases may subsequently liberate IGF-1 from such pools and cause greatly enhanced anabolic processes, contributing to cartilage repair (Purple *et al.*, 2002). It has been suggested that this mechanism may have an important role in maintenance and repair of articular cartilage and that age-related failure to organize this system may contribute to progressive articular cartilage degeneration (Martin *et al.*, 2000).

1.2.2 Extracellular matrix proteinases

Degradation of cartilage is a major feature of OA and RA. Major activities responsible for catabolism of proteoglycan (mainly aggrecan) and collagen (mainly Type II collagen) are attributable to zinc-dependent metalloproteinases (Flannery *et al.*, 2000) in particular the MMPs and aggrecanases.

1.2.2.1 Matrix metalloproteinases

The MMPs (Table 1.1) play an important role in the digestion of the extracellular matrix in both normal and degenerative articular cartilage. These enzymes target specific components of the extracellular matrix such as collagen, aggrecan, fibronectin, laminin, and elastin and may be activated through a number of signaling pathways (Holland and Mikos, 2003).

MMPs share a common domain structure comprising of a prodomain, which is a signal peptide for secretion, a prodomain to maintain latency, a catalytic domain containing a highly conserved zinc-binding site, a hinge region, and a C-terminal also known as a hemopexin-like domain (Fig. 1.6). Two family members, MMP-2 and MMP-9, have a gelatin-binding domain containing three fibronectin-type II repeats inserted into the catalytic domain (Szabo *et al.*, 2004).

I

Key to domains

signal peptide
propeptide
catalytic (metalloproteinase) domain
Zinc active site
2
C-6^-/./^

Figure 1.6: Diagram showing the domain structure of matrix metalloproteinases (MMPs). Some MMPs have additional domains located up- and/or down-stream of the catalytic domain such as: fibronectin domain; Type I and type II transmembrane domain; cytoplasmic domain; cysteine array region; and IgG-like domain. Modified from Visse and Nagase (2003).

Most MMPs are secreted as proenzymes into the extracellular milieu (Leppert *et al.*, 2001). However, six members of MMPs referred to as membrane-type MMPs (MT-MMPs) have recently been defined and were categorized in a

new subfamily due to their unique feature of a C-terminal anchor sequence (transmembrane domain) after the hemopexin domain that keeps them attached to the cell membrane (Leppert *et al.*, 2001). All MMPs are functional at neutral pH and are produced by a variety of cells including fibroblasts, neutrophils, eosinophils, macrophages, T-cells, chondrocytes and osteoblasts in response to inflammatory cytokines (Szabo *et al.*, 2004).

Physiology and pathology of MMPs

The modeling, disassembly and remodelling of connective tissue matrices by one or more MMPs involves secretion and proteolytic activation of the precursor MMPs (Szabo *et al.*, 2004). MMPs are synthesized as zymogens containing an N-terminal inhibitory propeptide sequence with a cysteine (Cys) that chelates the zinc (Zn^{2+}) ion and keeps the enzyme in a latent pro-form. Various factors can activate MMPs via a conformational change that disrupts the Cys- Zn^{2+} binding (cysteine switch) and leads to intermediate activation followed by autocatalytic cleavage of the propeptide from the core protein rendering the enzyme fully active (Leppert *et al.*, 2001; Szabo *et al.*, 2004).

Most MMPs are expressed only on demand through the action of cytokines, eicosanoids, growth factors and components of infectious pathogens, among others (Chien *et al.*, 2005; Leppert *et al.*, 2001) and the most prominent of these inducers are the pro-inflammatory cytokines IL-1 α/β and TNF- α/β .

MMPs contribute to joint destruction in at least two ways. First, they can directly enhance proteolytic degradation of the ECM of the cartilage and bone. The major MMPs implicated in the process are MMP-1, -2, -3, -9, -13 and -15, wherein MMP-15 activates proMMPs-2 and -13 and MMP-14 plays an important role in the activation of pro-MMP-2 in the rheumatoid synovial lining cell layer, suggesting involvement in the cartilage destruction of RA. Secondly, MMPs are important during angiogenesis, which is a prominent feature of RA (Szabo *et al.*, 2004).

Table 1.1: Summary of MMPs proteins

Group	MMP	Other name(s)	Special feature(s)	Function (s)
Collagenases	MMP-1 MMP-8 MMP-13 MMP-18 (Zenopus)		Specific cleavage site on collagens 3:4 th from N-terminus	Cleavage of collagens I, II and III; digestion of other ECM and other non-ECM
Gelatinases	MMP-2 MMP-9	Gelatinase A Gelatinase B	3 repeats of type II fibronectin domain inserted in the catalytic domain	Binding to collagens, gelatins and laminin; digestion of denatured collagens and gelatins; Osteogenesis
Stromelysins	MMP-3 MMP-10	Stromelysin 1 Stromelysin 2	MMP-3 more efficacious than MMP-10	Activation of a number of proMMPs; digestion of ECM
Matrilysins	MMP-7 MMP-26	Matrilysin-1 Matrilysin-2; Endometase	Lack of a hemopexin domain	Digestion of ECM; processing of surface molecules (e.g. Fas-ligand, pro-TNF- α)
MT-MMPs Type I transmembrane proteins	MMP-14 MMP-15 MMP-16 MMP-24	MT1-MMP MT2-MMP MT3-MMP MT5-MMP	Transmembrane domain MT5-MMP is brain specific and expressed mainly in the cerebellum	Activation of proMMP-2; Angiogenesis; digestion of ECM molecules including collagens I, II and III
Glycosylphosphatidylinositol (GPI) anchored proteins	MMP-17 MMP-25	MT4-MMP MT6-MMP		
Other MMPs	MMP-12	Metalloelastase	Expressed in macrophages	Macrophage migration; Digestion of elastin and other proteins
	MMP-19		Identified in the liver as cDIIA and as a T cell-derived autoantigen in RA	
	MMP-20	Enamelysin	Located within newly formed tooth enamel	Digestion of amelogenin
	MMP-22		First cloned from chicken fibroblasts and human homologue identified	Unknown
	MMP-23	Cysteine array MMP	Expressed mainly in reproductive tissues; lacks 'cysteine switch' motif and hemopexin domain; instead it has cysteine-rich domain followed by Ig-like domain	
	MMP-28	Epitysin	Expressed in keratinocytes	Tissue homeostasis and wound healing

Modified from Visse and Nagase (2003).

1.2.2.2 Aggrecanase proteins

Aggrecanases are members of ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) gene family (Tang, 2001). This is a novel group of extracellular proteases found in mammals including humans (Table 1.2). Although the existence of 'aggrecanase' was known for more than ten years based on its unique cleavage-site, it is only recently that two aggrecanases have been isolated from cartilage, cloned and expressed, designated ADAMTS-4 and ADAMTS-5 (aggrecanase-1 and -2, respectively) (Arner, 2002). The ADAMTS proteins consist of an N-terminal signal peptide and pro-domain and have the characteristic ADAM (a disintegrin and metalloprotease) -like catalytic domain with the zinc-binding motif, a disintegrin-like and a cysteine-rich domain (Arner, 2002; Tang, 2001). However, they differ from conventional ADAMs in that they lack the transmembrane and cytoplasmic domains exhibited by many of these proteins. They have a distinct feature, a thrombospondin type 1 (TSP-I)-repeat between the disintegrin-like and the cysteine-rich domain, a spacer region, followed by a varying number of TSP-1-like repeats at the C-terminus (Fig. 1.7). Since ADAMTS proteins lack transmembrane domains, they are therefore secreted into the ECM (Tang, 2001).

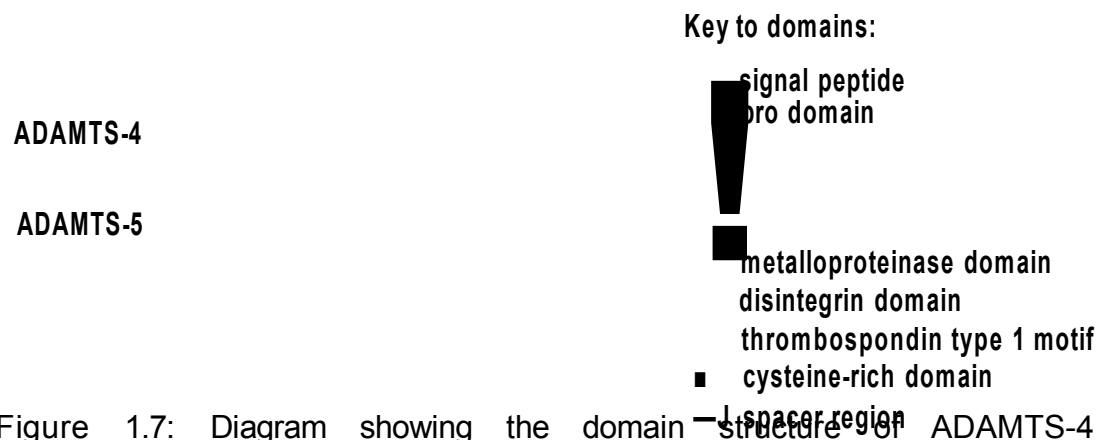


Figure 1.7: Diagram showing the domain structure of ADAMTS-4 (Aggrecanase-1) and ADAMTS-5 (Aggrecanase-2). Modified from Porter *et al.* (2005).

Both recombinant ADAMTS-4 and ADAMTS-5 cleave aggrecan within the IGD at a site not sensitive to MMPs. they also cleave aggrecan preferentially at four sites located in the CS-rich region between G2 and G3 (Tortorella *et al.*, 2000). ADAMTS-4 and/or ADAMTS-5 are the aggrecanases responsible for aggrecan catabolism following IL-1 and TNF treatment of bovine articular cartilage (Tortorella *et al.*, 2001).

Table 1.2: The human ADAMTS names and their function

ADAMTS	Other Names	Number of Tsp Motifs	Function
ADAMTS-1	METH-1; Aggrecanase-3; KIAA1346	3	Inflammation, angiogenesis, organ morphogenesis, tumour invasion
ADAMTS-2	Procollagen N-proteinase	4	Procollagen processing
ADAMTS-3	KIAA0366	4	Procollagen processing
ADAMTS-4	Aggrecanase-1; KIAA0688	1	Aggrecan, brevican, versican cleavage
ADAMTS-5	Aggrecanase-2; ADAMTS-11; Implantin	2	Aggrecan cleavage
ADAMTS-6		2	Unknown
ADAMTS-7		2	Unknown
ADAMTS-8	METH-2	2	Angiogenesis
ADAMTS-9	KIAA1312	13-15	Aggrecan, versican cleavage
ADAMTS-10		5	Unknown
ADAMTS-12		8	Unknown
ADAMTS-13	WVF-cleaving protease	8	von Willebrand factor cleavage
ADAMTS-14		4	Procollagen processing
ADAMTS-15			Aggrecan cleavage
ADAMTS-16			Unknown
ADAMTS-17			Unknown
ADAMTS-18			Unknown
ADAMTS-19			Unknown
ADAMTS-20			Unknown

Modified from Arner, 2002; Porter et al., 2005; and Tang, 2001

1.2.2.3 Inhibitors of extracellular matrix metalloproteinases

The activity of MMPs is strictly regulated by a group of inhibitors. Two known natural sources of MMP inhibition are the α_2 -macroglobulin (a large protein (≈ 750 kDa), produced by the liver) and the tissue inhibitors of matrix metalloproteinases (TIMPs) (Szabo *et al.*, 2004). TIMPs are more specific endogenous inhibitors of MMPs that consist of at least four members (TIMP-1, -2, -3 and -4) (Holland and Mikos, 2003; Szabo *et al.*, 2004). TIMPs control MMP activity by binding to the active site of MMPs. TIMPs may also bind to additional sites on latent MMPs (Holland and Mikos, 2003). TIMPs form noncovalent binding complexes in a 1:1 stoichiometry with pro and activated forms of MMPs and inhibit enzymatic activity. Although TIMPs bind tightly to most MMPs and are highly similar in their quaternary structure, their tissue distribution, regulation, and function are diverse. Aggrecanase activity is specifically inhibited by TIMP-3 (Kashiwaya *et al.*, 2001). Similar to the metalloproteinases, TIMPs are also regulated by a network of different signaling molecules. The TIMPs inhibit the MT-MMPs, however, unlike the soluble MMPs, the MT-MMPs exhibit significant differences in affinities for the various TIMPs (Szabo *et al.*, 2004). Elevated levels of MMPs and TIMPs are found in patients with OA and RA (Holland and Mikos, 2003).

1.3 Arthritis

1.3.1 Rheumatoid arthritis

RA is the commonest chronic inflammatory arthropathy (joint disease) of unknown origin with several extra-articular features suggesting systemic involvement and autoimmunity (Moller *et al.*, 2001; Scott *et al.*, 1998). RA affects about 1% of the population, with a marked female predominance (Sayah and English, 2005). The disease can occur at any age, but it is most common among those aged 40–70 years, its incidence increasing with age. The geographic distribution of rheumatoid arthritis is worldwide, with a notably low prevalence in rural Africa and high prevalence in specific tribes of Native Americans (Pima and Chippewa) (Lee and Weinblatt, 2001; Sayah and

English, 2005). In childhood, RA is rare and overlaps with other forms of chronic arthritis; it has a low incidence of 5/100 000 children/year and the prevalence of established disease is equally low (Scott *et al.*, 1998). The costs of managing RA are high and may cost the individual with the disease an average of £4000 p.a. in the UK (Scott *et al.*, 1998). There is no clear association between prevalence of rheumatoid arthritis and socioeconomic status (Lee and Weinblatt, 2001). Progressive bone and cartilage destruction, chronic synovial inflammation and extracellular matrix degradation leading to gross crippling joint deformation and pain characterize the disease (Szabo *et al.*, 2004).

1.3.1.1 Aetiology and pathology of RA

RA is a multi-system disorder with a complex aetiology (Carter, 1997). The most obvious manifestation is a destructive inflammatory arthritis but other systemic features contribute significantly to morbidity (Harney *et al.*, 2003). An inflamed synovium is central to the pathophysiology of RA (Fig. 1.8). Macrophages, fibroblast-like synoviocytes (FLS) and lymphocytes are the predominant cell types involved in RA synovitis (Moller *et al.*, 2001). It is histologically striking, showing pronounced angiogenesis; cellular hyperplasia; an influx of inflammatory leucocytes; and changes in the expression of cell-surface adhesion molecules, proteinases, proteinase inhibitors, and many cytokines. The synovial changes vary with disease progression (Lee and Weinblatt, 2001). The formation of locally invasive synovial tissue, pannus, is a characteristic feature of rheumatoid arthritis. This tissue is involved in the joint erosions (Fig. 1.8). Pannus is histologically distinct from other regions of the synovium and shows phases of progression. Initially, there is penetration of the cartilage by synovial pannus composed of mononuclear cells and fibroblasts with high-level expression of matrix metalloproteinases by synovial lining cells. In later phases of the disease, cellular pannus can be replaced by fibrous pannus comprised of a minimally vascularised layer of pannus cells and collagen overlying cartilage. The pannus cells are thought to arise from fibroblast-like cells (type B synoviocytes) (Lee and Weinblatt, 2001).

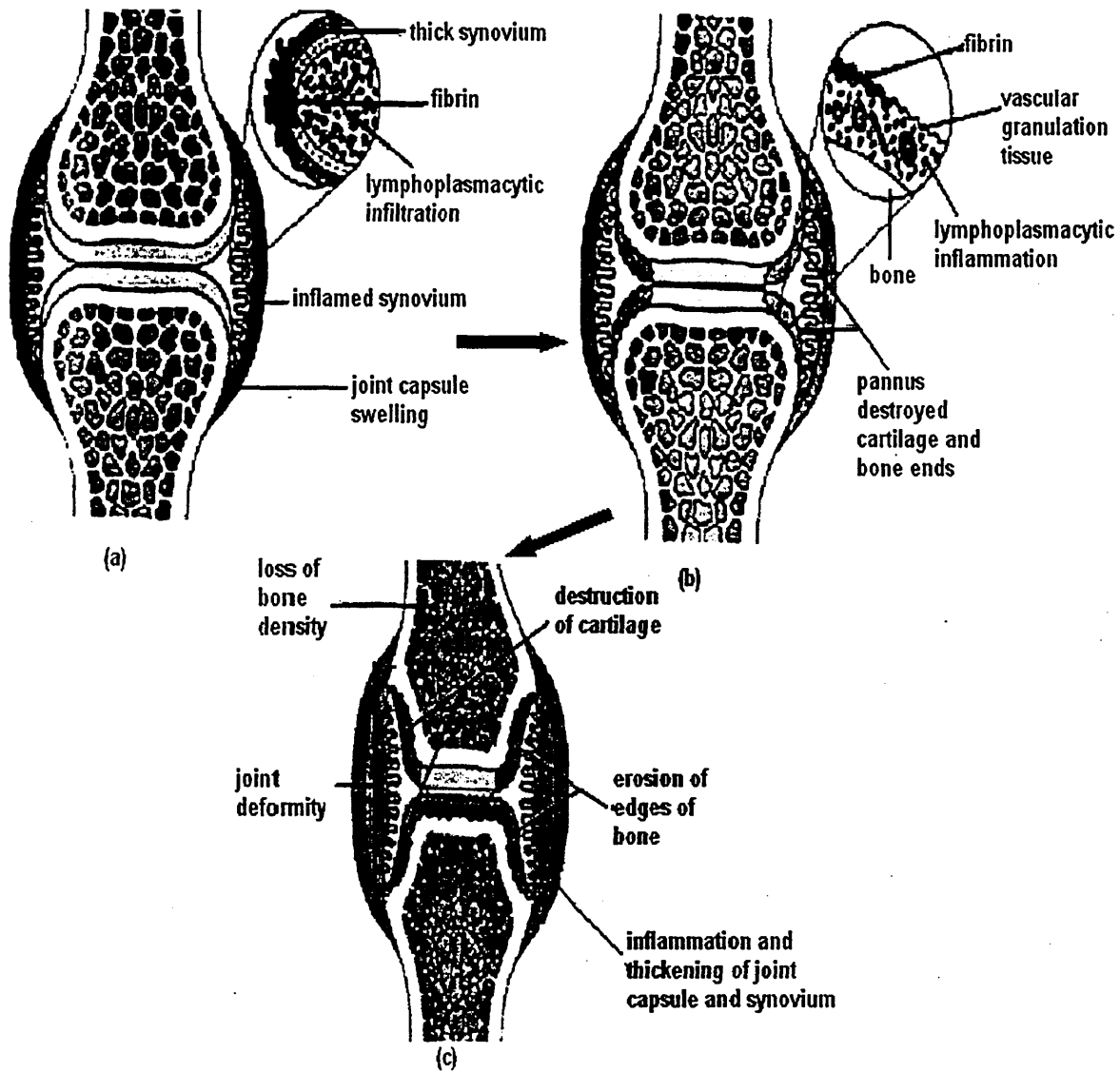


Figure 1.8: Diagrams showing pathological changes in RA. (a) Early stages of RA, the synovium are inflamed and the capsule and soft tissue around the joint are swollen. Lymphocytes, macrophages and synoviocytes are involved in synovitis. (b) Pannus is formed and it destroys the cartilage at the periphery of the joint. (c) Later stages there is continued inflammation and thickening of capsule and synovium as well as erosion of the cartilage and bone by the pannus and other catabolic factors. Joint deformity and loss of bone density may ensue. Modified from Stevens and Lowe (2000).

Cytokine-stimulated T cells, interacting with macrophages in the rheumatoid synovial membrane, may contribute to the continuous excessive production of

TNF- α observed in the RA joint, and to the imbalance of pro-inflammatory cytokines over anti-inflammatory cytokines (Sebbag *et al.*, 1997). Targeting the action of individual cytokines, for example, using antibody against TNF- α has been shown to be very effective in the treatment of RA. Blockage of TNF- α activity results in deactivation of the endothelium, manifested as reduced expression of adhesion molecules and chemoattractant cytokines, leading to diminished trafficking of inflammatory cells to synovial joints. In addition anti-TNF- α decreases circulating levels of the potent angiogenic cytokine vascular endothelial growth factor (VEGF), suggesting that new blood vessel formation, and hence the supply of nutrients to the growing synovial lesion, is also affected. These observations lend further support to the hypothesis that interruption of a component of the cytokine network in RA may modulate disease progression, and point the way towards the development of new therapeutic strategies for the treatment of chronic inflammatory disease states (Paleolog, 1997).

1.3.1.2 Clinical features of RA

1. Constitutional symptoms (fatigue, anorexia, weight loss, fever)
2. Symmetric polyarthritis-primarily of peripheral joints (joints of the hand-usually sparing the distal interphalangeal joints, almost any diarthrodial joint)
3. Morning stiffness-greater than 1 hr
4. Erosive arthritis (loss of marginal aspects of the bones)
5. Deformity and decreased range of motion (ROM)
6. Rheumatoid nodules (subcutaneous masses - most common site being the olecranon bursa [elbow] however they can occur elsewhere and usually indicative of an active or more severe disease)
7. Extraarticular manifestations in RA may involve organs such heart [pericarditis], lungs [pleuritis], eyes, blood vessels and nervous system).

(Carter, 1997)

1.3.1.3 Genetics of RA

Over the years, the only area consistently shown to be associated with RA has been the major histocompatibility complex (MHC), which is located on chromosome 6 (6p21.3). This region contains about 220 genes, and about 40% of these have an immune function. The MHC is made up of three regions known as class I, II and III. Human leukocyte antigens (HLA) DR and DQ are located in the class II region and present antigens to T cells (Cornelis, 2003; Harney *et al.*, 2003). HLA-DR alleles associated with RA include those belonging to the HLA-DR4; HLA-DR1 and HLA-DR10 (Cornelis, 2003). It has also been known for years that HLA-DQ3 and DQ5 alleles are associated with RA (Harney *et al.*, 2003). DR4 alleles and related haplotypes are associated with severe, persistent, rheumatoid factor-positive polyarthritis with extraarticular manifestations. However, the question whether DR4 influences disease severity or susceptibility is still a matter of debate. Even less understood is the actual contribution of DR1 and DR10 alleles and related haplotypes to RA (Zanelli *et al.*, 2000). However increased frequencies of DR4 were observed in males and DR1 in females with RA (Sherritt *et al.*, 1996).

Genetic factors however appear to make a limited contribution to the development of RA as implied by the statistics that state that first-degree relatives of RA patients have an estimated risk of approximately 4 % and identical twins have approximately 12% risk rate. This suggests a role for other factors, probably related to the environment (Sayah and English, 2005).

1.3.1.4 Disease management

The previous therapeutic approach, termed the therapeutic pyramid, involved initial management of RA with the non-steroidal anti-inflammatory drugs (NSAIDs) for several years until clear evidence of erosions was seen. The disease modifying anti-rheumatic drugs (DMARDs) were then added individually in slow succession as the disease progressed. This kind of treatment has been replaced with early initiation of DMARDs and combination

DMARD therapy in patients with the potential for progressive disease (Sayah and English, 2005). DMARDs slow the disease process by modifying the immune system and thus decreasing inflammation, however they have significant side effects. DMARDs include methotrexate (MTX), gold, hydroxychloroquine, sulfasalazine, cyclosporine, and azathioprine. MTX is now being used as base for combination therapy regimens with gold, hydroxychloroquine, sulfasalazine, cyclosporine, azathioprine and newly approved drugs such as leflunomide (anti-inflammatory), etanercept and infliximab (antibodies against TNF- α), and celecoxib (selective COX-2 inhibitor) (Sayah and English, 2005).

The efficacy of fish oils such as cod liver oil, in the diet has been demonstrated in several clinical trials, animal feeding experiments and *in vitro* models that mimic cartilage destruction in arthritic disease (Curtis *et al.*, 2004). A variety of other agents, such as glucocorticoids and nutraceuticals such as glucosamine, chondroitin sulfate, hyaluronic acid, green tea, also appear to modify the symptoms and course of arthritis (Ulrich-Vinther *et al.*, 2003). However, in most cases, scientific studies are still being carried out to identify their mechanisms of action at the cellular and molecular levels (Curtis *et al.*, 2004).

1.3.2 Osteoarthritis

Although osteoarthritis (OA) is generally more benign than RA, it is still the most common joint disorder and a major contributor to disability in the elderly (Arjmandi *et al.*, 2004; Scott *et al.*, 1998). It is more common in women than men (Scott *et al.*, 1998). OA has high prevalence (6–12% of the adult population) (Auw Yang *et al.*, 2004) although true prevalence is difficult to measure as clinical definitions rely on the presence of pain and as such many cases of asymptomatic disease are missed (Salter, 2002). Its prevalence increases with age (Fautrel *et al.*, 2005) but age per se does not cause OA (Salter, 2002). Radiographic assessments suggest that OA affects roughly 80% of the population over 75 years (Blumenkrantz *et al.*, 2004; Salter, 2002). The efficacy of articular cartilage repair also decreases with increasing

age. Martin and Bulkwalter (2003) reported that chondrocytes as they age, synthesize smaller, less uniform aggrecan molecules and less functional link proteins, their mitotic and synthetic activity decline, and their responsiveness to anabolic mechanical stimuli and growth factors decreases. Therefore chondrocyte senescence contributes to the age-related increase in the prevalence of OA and decrease in the efficacy of cartilage repair (Martin and Bulkwalter, 2003).

Although there is a lack of agreement regarding the definition of OA, it is generally viewed as a degenerative disorder (Arjmandi *et al.*, 2004) in which bone and cartilage morphological and biochemical changes cause abnormal biomechanical loading patterns, leading to joint deformity, progressively increasing pain, (morning) stiffness, crepitus, and decreased mobility or limited range of motion of the joint involved (Auw Yang *et al.*, 2004; Blumenkrantz *et al.*, 2004). Typically, the degenerative changes are accompanied by a local inflammatory component that accelerates the joint destruction (Arjmandi *et al.*, 2004), although rather limited due to the avascular nature of the cartilage tissue (Salter, 2002).

1.3.2.1 OA aetiology and pathology

OA is usually classified as primary (idiopathic) or secondary when it follows some clearly defined predisposing disorder. Mechanical factors play a role in all types of primary, as well as secondary OA. Joint failure results from an imbalance between the combinations of mechanical stresses and catabolic processes acting on the joint and the capacity of its tissues to withstand the strains and repair the damage. It is likely that in the majority of patients with primary, idiopathic OA multiple genetic and environmental factors are contributing to the pathogenesis and progression of joint failure (Blanco *et al.*, 2004). Risk factors for OA include factors that increase joint vulnerability (malalignment, muscle weakness, genetic and ethnic predispositions, ageing) and those that cause excessive loading (obesity; certain physical activities) (Felson and Schurman, 2004). Excessive mechanical stresses and major trauma are recognized as being important causes of disease. There is an

increased incidence of degenerative joint disease in certain occupations such as farmers, coalminers and shipyard workers and following injuries to joints such as cruciate ligament rupture in sportsmen (Salter, 2002). Mechanical factors, when optimally applied, are important in maintaining cartilage in a healthy state and moderate regular use of a joint has a negligible risk of osteoarthritis (Salter, 2002). This may help explain why strenuous repetitive usage of joints for short periods is likely to increase the incidence of OA when there is associated injury and loss of the normal joint biomechanics (Salter, 2002). Articular cartilage has a limited ability to heal after trauma or maintain its function with increasing age due to the avascular nature of the tissue and the limited ability of chondrocytes to migrate within the tissue (Auw Yang *et al.*, 2004). Obesity has also been found to be associated with an increase in OA and although this may be explained by biomechanical factors, a metabolic role for fat in disease pathogenesis has been suggested (Salter, 2002).

At the molecular level, OA is characterized by an imbalance between chondrocyte anabolism and catabolism. Disruption of chondrocyte homeostasis primarily affects the cartilage ECM, which is responsible for the biomechanical properties of the tissue (Malemud, 2004). Morphological changes observed in OA include cartilage erosion as well as a variable degree of synovial inflammation (Fernandes *et al.*, 2002) (Fig. 1.9). The disease processes not only affect the articular cartilage, but involve the entire joint, including the subchondral bone, ligaments, capsule, synovial membrane, and periarticular muscles. Ultimately, the articular cartilage degenerates with fibrillation, fissures, ulceration and full thickness loss of the joint surface (Salter, 2002). As cartilage function is compromised, the loading of the underlying bone is altered. This is followed by an increase in bone remodelling resulting in bone thickening and joint deformity occurs caused by benign hypertrophic reactions at the edges of the joints (osteophytes) (Fig. 1.9) (Auw Yang *et al.*, 2004). Loss of cartilage leads to bone-to-bone articulation (Fig. 1.9) which is associated with much pain. Pain in OA usually increases after exercise and decreases at rest and in more severe cases, pain can even occur after minimal usage or at rest (Auw Yang *et al.*, 2004). OA can also occur without pain (Felson and Schurman, 2004). Stiffness

occurs mainly in the morning after sleep and after prolonged rest. It usually disappears within 10 min after minimal motion (Auw Yang *et al.*, 2004). Decreased mobility is caused by pain; joint destruction and inflammation of the soft tissue surrounding the involved joint (Auw Yang *et al.*, 2004).

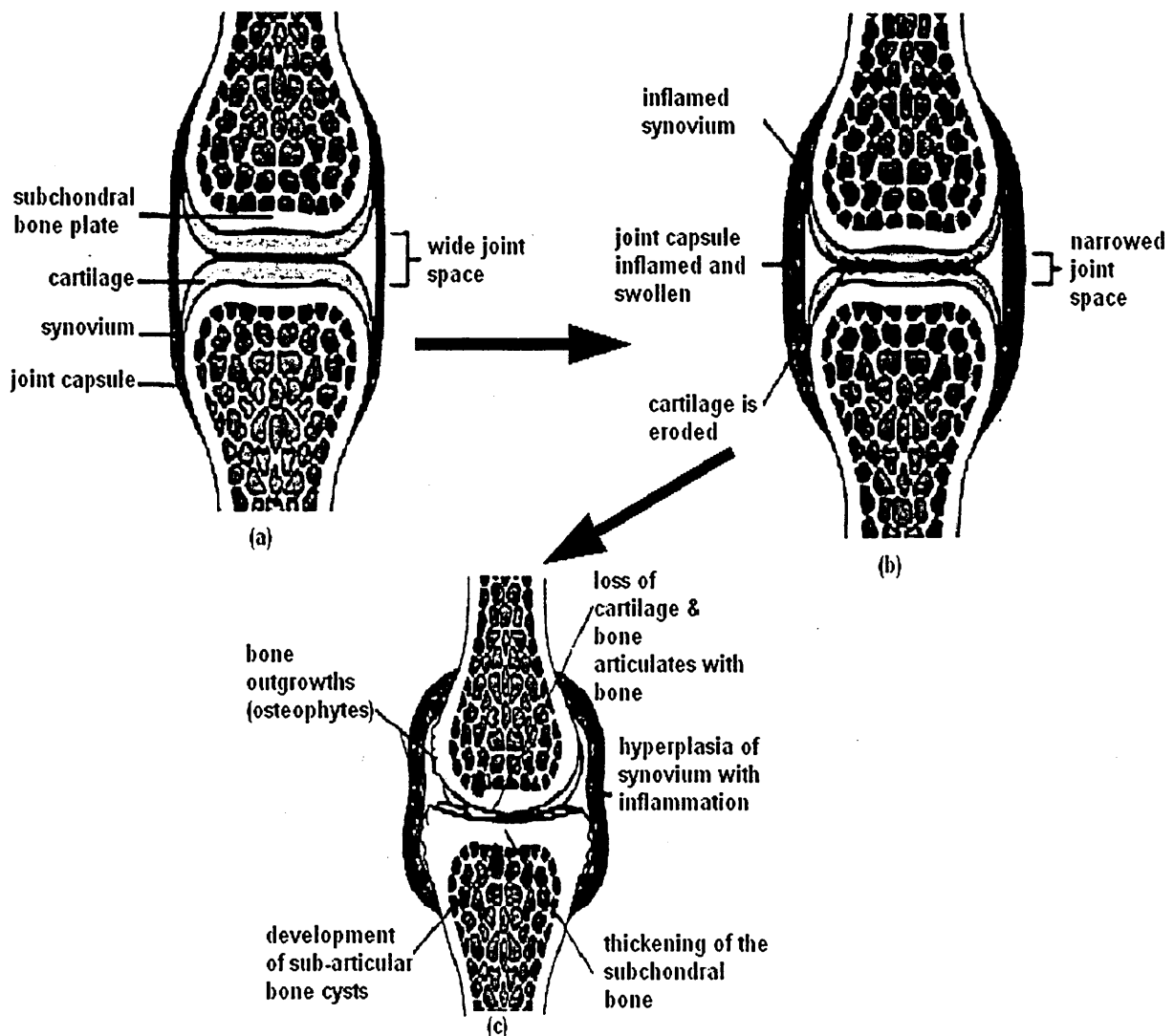


Figure 1.9: Diagrams showing pathological changes in OA. (a) Normal joint. (b) In the early stages of OA, the joint capsule and the synovium are inflamed and swollen (oedema). Cartilage disintegrates and is eroded leading to narrowed joint space. (c) In the later stages of the disease, inflammation of synovium leads to hyperplasia, loss of cartilage leads to bone-to-bone articulation (eburnation). The deformation of subchondral bone follows as seen in its thickening and development of osteophytes (bone outgrowths) and bone cysts. Modified from Stevens and Lowe (2000).

A number of pro-inflammatory cytokines have been implicated in the OA process of cartilage destruction including IL-1, TNF- α , IL-6, and IL-17 (Fernandes *et al.*, 2002; Malesud, 2004). The primary role of these cytokines is to modulate the expression of matrix metalloproteinases and cartilage ECM proteins. IL-1 and TNF- α produced by activated synoviocytes, mononuclear cells or by articular cartilage itself significantly up-regulate MMP gene expression. The cytokines were shown to significantly up-regulate MMP-3 steady-state mRNA derived from human synovium and chondrocytes (Fernandes *et al.*, 2002). Cytokines also impair chondrocyte compensatory synthesis pathways required to restore the integrity of the degraded ECM. Chondrocytes in OA cartilage appear unable to respond to anabolic factors such as IGF-1 (Malesud, 2004). IL-1 α was found to increase the expression of IGFBPs and also reduce the IGF-I bioactivity, suggesting that locally produced IGFBPs mediated the reduction of the IGF-I activity (Sunic *et al.*, 1998). IGFBP-3 and -5 expression and production has been shown to be elevated in osteoarthritic cartilage and they sequester IGF-I in osteoarthritic cartilage, thus reducing the anabolic stimulation of this tissue and contributing to the net loss of cartilage in this disease (Olney *et al.*, 1996). Furthermore, in OA synovium, a relative deficit in the production of natural antagonists of the IL-1 receptor, IL-1Ra, has been demonstrated and it has been suggested this may be related to an excess production of nitric oxide in OA tissues. This, coupled with an upregulation in the receptor level, has been shown to be an additional enhancer of the catabolic effect of IL-1 in this disease (Fernandes *et al.*, 2002).

Elevated levels of nitrite, a stable end-product of NO metabolism were found in serum and synovial fluid samples from arthritis patients, including OA patients (Jang and Murrell, 1998). In OA tissue, iNOS is the enzyme that is responsible for the increased production of NO (Alderton *et al.*, 2001). In articular chondrocytes and cartilage, NO plays a regulatory role in the activation of metalloproteinases (Murrell *et al.*, 1995) and thus may be involved in cartilage catabolism. In addition, induction of synovial and chondrocyte COX-2/ mPGES-1-produced PGE₂ has been shown to be one mechanism by which IL-1 modulates cartilage proteoglycan degradation in

OA (Hardy *et al.*, 2002). This synthesis was shown to be regulated by IL-1 acting via the ERK-1/2 and p38 MAPK signalling pathways (Masuko-Hongo *et al.*, 2004) and perhaps, cAMP-dependent protein kinase (Faour *et al.*, 2001). The synthesis of cyclo-oxygenases, nitric oxide synthase, and neutral proteinases is increased in inflammatory synovitis, and these enzymes might mediate parts of the disease process (Lee and Weinblatt, 2001).

Decreased proteoglycan content and increased water content in articular cartilage, as well as, elevated concentrations of proteoglycans and fibronectin fragments in the synovial fluid are characteristic of osteoarthritis due to the upregulation of MMPs production by chondrocytes (Holland and Mikos, 2003).

There is a chronic, inflammatory form of osteoarthritis which involves both the proximal and distal rows of finger joints and leads ultimately to marked deformity of the digits. This is referred to as erosive OA (EOA) and some of its clinical features include abrupt onset of pain, swelling, redness, warmth and limited function of interphalangeal joints of the hands (hallmarks of inflammation) (Punzi *et al.*, 2004). EOA may lead to joint deformities such as formation of Heberden's and Bouchard's nodes which are present at distal and proximal interphalangeal joints, respectively (Greenspan, 2003; Punzi *et al.*, 2004).

1.3.2.2 Genetic influences in OA

Genetic factors are important in some forms of OA as has been recognized for some time in cases of idiopathic OA associated with Heberden's nodes (nodal OA) (Salter, 2002) Sisters of females with Heberden's nodes in the distal and proximal interphalangeal joints, and carpometacarpal joints of the hand, were three times more likely than the general population to exhibit nodal OA at age 50 (Simonet, 2002). In subsequent studies of generalised OA of the hand, the frequency of OA was nearly twice as high in OA pedigrees compared with the general population. Familial studies reveal a higher incidence of OA among monozygotic versus dizygotic twins, a higher prevalence of OA in siblings of affected individuals than in the normal

population, and a higher prevalence of OA in first-degree relatives (siblings, parents, and offspring) of affected individuals than in the normal population. Together, these studies confirm a strong genetic influence on the disease (Simonet, 2002). Type II collagen mutations have been demonstrated in some families with early onset OA and associations with the vitamin D receptor gene, the type I collagen gene COL1A1, and the oestrogen receptor gene in idiopathic osteoarthritis are being studied (Salter, 2002). It is likely that common OA, like some other diseases of ageing, is a polygenic disease, where common alleles of multiple genes each contribute to a small increased risk either individually or by interaction with each other or with environmental factors (Simonet, 2002).

1.3.2.3 OA management

Goals are to prevent or retard further damage to joints, to manage pain and stiffness, and maintain mobility. First, protection of joints from additional trauma is important- in particular eliminating those activities that increase the load-bearing strain to an affected joint. Canes and walkers can significantly decrease the weight load on knees and hips. Weight reduction if the person is overweight, can greatly decrease the load placed on knees and hips. Secondly, physical therapy is important for relieving pain and preserving muscle strength and range of motion (ROM). Use of ice and heat on involved joints may also provide temporary relief of pain. Drug therapy designed to control pain and any synovitis is also one of the means of managing OA. Over-the-counter (OTC) analgesic drugs such as aspirin and ibuprofen are usually adequate for pain relief. The drugs have the added advantage of controlling the synovitis. Other NSAIDS are used as well for pain and synovitis control. Adverse effects of the drugs are generally common in older persons so drug therapy should be considered carefully since many in the age group have OA. DMARDs are not used in OA because it is not a systemic disorder. Oral corticosteroids are contraindicated, since they are generally not effective in improving symptoms and their toxic potential make their use risky. Intraarticular injections can provide relief of synovitis but if

used too frequently, the agents deplete the components of the cartilage and can accelerate the arthritic progression.

A final option is surgical treatment that is designed to remove loose bodies; repair damaged supporting tissues or to replace the entire joint (Arjmandi *et al.*, 2004; Carter, 1997)

1.4 Inflammatory mediators associated with arthritis

As mentioned above, in OA proinflammatory cytokines including IL-1 have been implicated as important mediators in the disease. In response to IL-1, chondrocytes upregulate the production of nitric oxide (NO) and prostaglandin E₂ (PGE₂), two factors that have been shown to induce a number of the cellular changes associated with OA (Hedbom and Hauselmann, 2002).

1.4.1 Nitric Oxide

NO is a very small molecule of 30 Da (Coleman, 2001) synthesized from the L-arginine by a family of enzymes, the NO synthases. Its size and its unpaired electron make it a highly reactive and locally diffusible free radical (Murrell *et al.*, 1996). Research on the biological roles of nitric oxide has revealed that it functions as an important signal and effector molecule in a variety of physiologic and pathologic settings (Stuehr, 1997).

Synthesis

NO is synthesised universally from L-arginine and molecular oxygen by an enzymatic process that utilises electrons donated by NADPH. The NO synthase (NOS) enzymes convert L-arginine to NO and L-citrulline via the intermediate *N*-hydroxy-L-arginine. One molecule of L-arginine produces one molecule of NO, the nitrogen atom of the latter deriving from a terminal guanidino group of the arginine side chain (Coleman, 2001). There are three known isoforms of the enzyme, named neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), which differ in their dependence on Ca²⁺, as well as in their expression and activities. These unique features give

rise to the distinct subcellular localisations and mechanistic features which are responsible for the physiological and pathophysiological roles of each isoform (Andrew and Mayer, 1999). The NOS isoforms are all dimeric, bi-domain enzymes that contain iron protoporphyrin IX, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH₄) as bound prosthetic groups (Stuehr, 1997). Cytokine-inducible NO synthase (iNOS) contains an oxygenase domain that binds haem, BH₄, and L-arginine, and a reductase domain that binds FAD, FMN, calmodulin, and NADPH. Dimerisation of two oxygenase domains allows electrons to transfer from the flavins to the haem irons, which enables O₂ binding and NO synthesis from L-arginine (Siddhanta *et al.*, 1998). The haem is essential for dimerisation as well as NO production and the BH₄ also affects dimerisation and electron transfer, although its full role in catalysis remains to be determined (Andrew and Mayer, 1999). NO presence in cells or tissue can be determined indirectly, through the measurement of its stable metabolites, nitrite (NO₂⁻) and nitrate (NO₃⁻) which act as marker molecules (Jang and Murrell, 1998).

NO and cGMP production

Cyclic guanosine 3',5'-monophosphate (cGMP) is an important second messenger molecule, which is formed from guanosine triphosphate (GTP) in a reaction catalyzed by guanylyl cyclase (GC). There are two families of cGMP-producing GCs, the transmembrane receptor class and the soluble class. NO increases the activity of soluble GC by binding to its haem iron and thereby raising the cGMP levels in the cell, which was the first signalling function of NO to be discovered (Schindler and Bogdan, 2001).

NO and cartilage metabolism

The iNOS is thought to play a role during host defense and immunological reactions (Murrell *et al.*, 1995). Articular chondrocytes are the major source of inducible nitric oxide synthase and nitric oxide during inflammation or infection of a joint. NO plays a regulatory role in the activation of metalloproteinases

(Murrell *et al.*, 1996). Involvement of NO in arthritis was first demonstrated when elevated levels of nitrite, a stable end product of NO metabolism were found in serum and synovial fluid samples from RA and OA patients (Jang and Murrell, 1998)

1.4.2 Eicosanoids

Prostaglandins and other structurally related molecules, prostacyclins, thromboxanes and leukotrienes are referred to as eicosanoids because they contain 20 carbon atoms. They act locally or near site of synthesis in the body. They are derived from a common precursor arachidonic acid (Hames and Hooper, 2000). Prostaglandins are not stored in cells but are synthesized and released immediately (Foegh and Ramwell, 2004). Prostaglandins produce a number of pharmacological effects. One of the most important effects is their activity as natural mediators of inflammation. Administration of PGE₂ induces signs of inflammation that include: redness, heat, swelling and oedema; and in the presence of histamine and bradykinin, enhances both intensity and duration of pain caused by these two agents (Glew, 2002). LTB₄ regulates neutrophil and eosinophil function and is a mediator of hypersensitivity, inflammation, secretion (e.g. insulin), cell movement, cell growth and calcium fluxes (Glew, 2002).

1.4.2.1 Prostaglandin E₂

Prostaglandin E₂ biosynthesis

Two unique cyclooxygenase (COX) isoenzymes convert arachidonic acid into prostaglandin G₂ (PGG₂), namely prostaglandin Synthase-1 (PGS-1) also known as COX-1 and PGS-2 also referred to as COX-2. COX -1 is constitutively expressed, widely distributed and has "housekeeping" functions whilst COX-2 expression depends on stimulus and is an immediate early response gene product in inflammatory and immune cells. COX-2 expression is stimulated by growth factors, tumour promoters, cytokines and lipopolysaccharide (LPS) (Foegh and Ramwell, 2004).

The PGSs catalyse both the oxygenation of arachidonic acid to PGG₂ and the reduction of PGG₂ to prostaglandin endoperoxide H₂ (PGH₂) (Glew, 2002; Masuko-Hongo *et al.*, 2004). Prostaglandin E synthase (PGES) in turn converts PGH₂ to prostaglandin E₂ (PGE₂) (Glew, 2002; Hames and Hooper, 2000; Masuko-Hongo *et al.*, 2004) (Fig. 1.10). Three distinct types of PGES have been identified and characterised to date namely: the cytosolic type, cPGES and two membrane-associated PGES-1 and -2 (mPGES-1 and mPGES-2, respectively) (Masuko-Hongo *et al.*, 2004).

PGE₂ production and cartilage metabolism

Tanioka *et al.* (2000) identified cPGES, the terminal enzyme of the cyclooxygenase (COX)-1-mediated PGE₂ biosynthetic pathway. Thus, functional coupling between COX-1 and cPGES/p23 may contribute to production of the PGE₂ that plays a role in maintenance of tissue homeostasis. Tanikawa *et al.* (2002) identified and characterised the mPGES with a molecular weight of approximately 33 kDa. The mPGES-1 is the inducible terminal synthase in the COX-2-dependent PGE₂ production pathway (Claveau *et al.*, 2003). The production of mPGES is stimulated by proinflammatory agents in several cells and tissues including rheumatoid synovial cells (Masuko-Hongo *et al.*, 2004). The mPGES-1 has been shown to be up-regulated throughout the development of adjuvant-induced arthritis and it has been suggested that it plays a major role in the elevated production of PGE₂ in this arthritis model (Claveau *et al.*, 2003). IL-1 stimulates the expression of secreted phospholipase A₂ and COX-2 genes in articular chondrocytes, resulting in increased PGE₂ production (Masuko-Hongo *et al.*, 2004). The Masuko-Hongo group (2004) also reported overexpression of mPGES-1, but not cPGES, in OA cartilage and in human articular chondrocytes stimulated with IL-1. Chondrocytes from OA cartilage were also shown to synthesize mPGES-1, and that this synthesis is regulated by IL-1 acting via the ERK-1/2 and p38 MAPK signaling pathways (Masuko-Hongo *et al.*, 2004). Although PGE₂ can be produced by both COX-2/mPGES and COX-1/cPGES pathways, the COX-2/mPGES pathway could be of

importance for increased PGE₂ production at sites of inflammation (Kojima et al., 2003).

1.4.2.2 Leukotriene B₄ biosynthesis

Lipoxygenase (LOX) is a dioxygenase, one of which is 5-LOX that produces leukotrienes that are important in humans and are involved in mediation of inflammatory disorders. Leukotrienes contain at least 3 conjugated double bonds. 5-LOX oxygenates AA to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE). 5-HPETE is converted to LTA₄ which in turn is converted to LTB₄ catalysed by LTB₄ synthase (LTA₄ hydrolase) which adds water to the double bond between C11 and C12 (Glew, 2002; Hames and Hooper, 2000) (see Fig. 1.10).

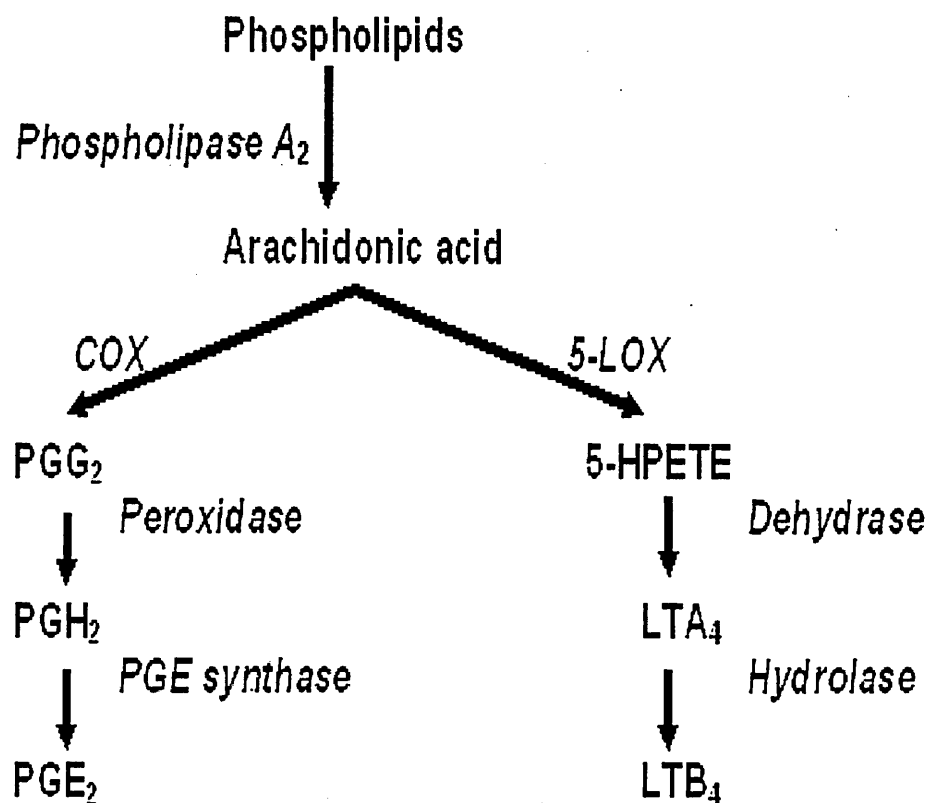


Figure 1.10: Pathways for PGE₂ and LTB₄ biosynthesis

1.5 Cannabinoids

1.5.1 History of *Cannabis sativa* and cannabinoids use

Cannabis sativa (Fig. 1.11), a plant belonging to the family of *Cannabaceae* (Pars and Howes, 1977) represents one of the oldest cultivated, economic plant providing fibre, edible seeds and drug resin (Philippe, 2004).

Figure 1.11: The *Cannabis sativa* plants. Adapted from Brown (1998).

Cannabis sativa appears to have originated from Central Asia, north of the Himalayas (Peters and Nahas, 1999). It was cultivated in China for its fibre and oil in its seeds (Peter and Nahas, 1999). The first evidence of the medicinal use of *Cannabis* comes from China. In 2737 BC, the Chinese emperor Shen-nung published a pharmacobotanical compendium, the *Pen-ts'ao Ching*, describing the use of *Cannabis* in the treatment of a variety of diseases including malaria, constipation, rheumatic pains, "absentmindedness" and female disorders (Calixto *et al.*, 2000; Guzman *et al.*, 2001; Straus, 2000). Shen-nung in his monograph concluded that cannabis "undoes rheumatism" (Straus, 2000). From China its use spread to Persia and Assyria in the 8th century BC (Peters and Nahas, 1999). The

Greeks and Romans also cultivated *Cannabis* for its fibre and manufactured ropes and sails (Peters and Nahas, 1999). *Cannabis* was also used for medicinal purposes in the ancient Egyptian civilisation (Guzman *et al.*, 2001).

The next widespread use of *Cannabis* occurred in India where it was also cultivated. Its use spread further to the Middle East. It started in the wake of the rise of Islam and met little cultural opposition, since Muhammad forbids the use of wine but not explicitly the *Cannabis* derivatives – hashish (Arabic name for “grass”) (Peters and Nahas, 1999). The Arabs introduced *Cannabis* preparations into North Africa from Egypt to Tunisia in the 9th – 20th centuries. During these centuries, studies done by the Muslim scholars and debates through the centuries led to prohibition of *Cannabis*, except for legitimate medicinal purposes. This religious prohibition still prevails in Islamic countries under penalty of law, which in some countries include the death sentence (Peters and Nahas, 1999).

In Europe, *Cannabis* was introduced by Napoleon Bonaparte’s troops when returning from Egypt (Berdyshev, 2000). In England, the history of the therapeutic use *Cannabis* dates as far back as the 1500s. It was reported then, that “water of hempe” freshly prepared from dried leaves of the plant were recommended for headaches and “for all here wheresoe’er it be” (Crawford, 2002). This was later supported by the founding father of modern English herbalism, Culpeper. He also commented on the usefulness of *Cannabis* to those with jaundice accompanied by a fever and recommended its use for diarrhoea, colic and rheumatic pain. He also recommended fresh *Cannabis* root mixed with oil and butter for burns and its seeds “seethed in milk” for coughs (Crawford, 2002). William O’Shaughnessy, an army physician in Calcutta, India during the 1830s found *Cannabis* to be effective in the treatment of rabies, rheumatism, epilepsy, and tetanus and as an analgesic (Calixto *et al.*, 2000; Peters and Nahas, 1999). Following the experiments carried out by O’Shaughnessy and as a result of the publication of his extensive article on the use of *Cannabis* in various medical conditions (Calixto *et al.*, 2000; Crawford, 2002), *Cannabis* was introduced into

European medicine and subsequently into other areas of western medicine including the US (Calixto *et al.*, 2000).

The medicinal use of *Cannabis* switched from the leaves, roots and seeds to the concentrated resin, rich in delta-9-tetrahydrocannabinoid (Δ^9 -THC) and other cannabinoids, from the Indian variety of the plant (Crawford, 2002). *Cannabis* preparations such as 'tincture and extract of *Cannabis*' were recognised for a long time as official drugs and were included in the British and US pharmacopoeias from the 1850s (Berdyshev, 2000; Calixto *et al.*, 2000). In Britain, *Cannabis* continued to be regarded as a benevolent therapeutic plant capable of soothing pain, calming anxiety, promoting appetite and digestion and relieving both headaches and nervous convulsions (Crawford, 2000). According to the records, dosages given were not strong enough to produce psychoactive effects. However many of the physicians noted the extreme variability in potency of *Cannabis* extracts and the difficulty in obtaining replicable results (Peters and Nahas, 1999). Thus *Cannabis* started to lose medical attention as new, pure pharmaceutical drugs began to appear such as aspirin and barbiturates that could be given in standard doses with reliable effects (Berdyshev, 2000).

As the medicinal use of *Cannabis* dwindled, its recreational use increased. In western countries, this abuse and perceived behavioural and addictive effects of *Cannabis* led to withdrawal of its preparations from the US pharmacopoeia and laws were passed prohibiting its use in the 1930s and 1940s (BMA report, 1997; Straus, 2000). In Britain, it remained permissible for doctors to prescribe *Cannabis* preparations until 1971 (BMA report, 1997).

Cannabis is by far the most widely used illegal drug in the Western world, with an estimated 20 million regular users in North America and Europe. Many thousands of patients with AIDS, multiple sclerosis, and other illnesses are also illegally self-medicating with *Cannabis* in the belief that it provides them with a therapeutic benefit (Iversen, 1999). The scientific study on *Cannabis* however, did not commence until the early 1960s, when Mechoulam and his colleague identified Δ^9 -THC (Guzman *et al.*, 2001). This led to growth in the

production of synthetic cannabinoid analogues and the discovery of cannabinoid receptors (Berdyshev, 2000).

1.5.2 Cannabinoid receptors

In the 1980s, studies on cannabinoids revealed that: the effects of cannabinoids are stereoselective; the psychoactivity of cannabinoids is not related to their lipophilicity, thus ruling out the hypothesis that states that cannabinoids because of their high hydrophobicity act by disrupting the membrane integrity; and the cannabinoid concentrations that are able to disrupt membranes integrity are excessively high. Hence these data suggested that the cannabinoids mediate their action through specific receptors (Guzman *et al.*, 2001). The first pharmacological evidence for cannabinoid receptors was reported by Devane *et al.* (1988) who showed the binding of the radioactive synthetic cannabinoid CP-55,940 to rat brain homogenates. In 1990, the first cannabinoid receptor was isolated and cloned from a cortical rat brain cDNA library (Matsuda *et al.*, 1990). Subsequently, human and mouse brain-type cannabinoid receptors were cloned (Berdyshev, 2000). A second subtype of cannabinoid receptors was subsequently isolated and cloned from a human promyelocytic HL-60 cell line (Munro *et al.*, 1993). According to the International Union of Pharmacology Subcommittee on Cannabinoid Receptors, these two cannabinoid receptors are termed, CB₁ and CB₂, respectively (Howlett *et al.*, 1998). Both receptors are members of the 7-transmembrane-helices, G-protein coupled receptor superfamily, and share 44% homology with each other (68% nucleotide identity within transmembrane regions) (Guzman *et al.*, 2001) (Fig. 1.12).

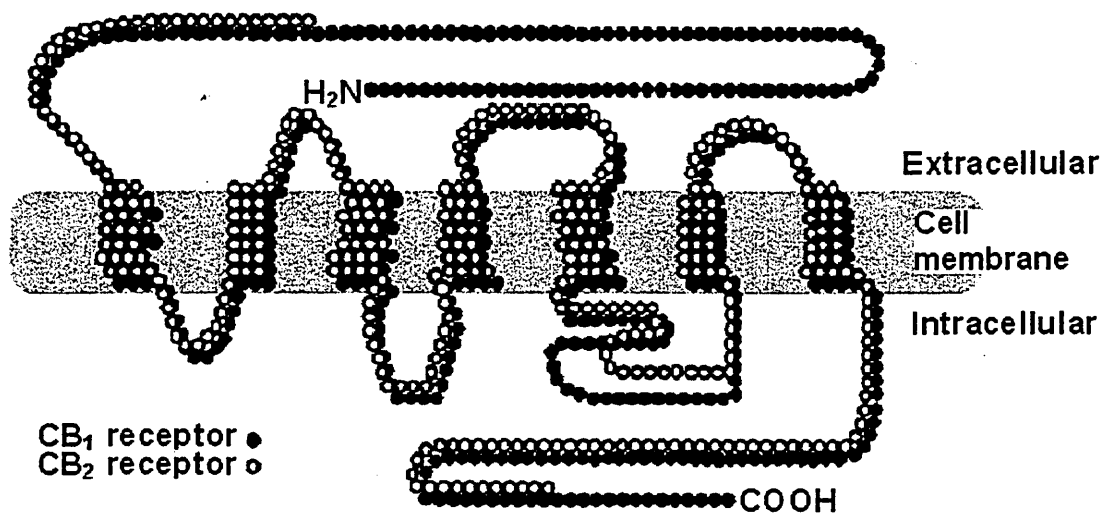


Figure 1.12: The diagram showing CB₁ and CB₂ cannabinoid receptors. The small circles represent amino acid residues. Modified from Joy *et al.* (1999).

In spite of this difference between the two types of cannabinoid receptors, most natural cannabinoids as well as endogenous ligands for cannabinoid receptors showed similar binding affinity to both receptor subtypes (Pertwee, 1999). Tissue expression of these receptors is rather different. CB₁ cannabinoid receptor also known as a central cannabinoid receptor is primarily found in the central nervous system; although it is detected at low levels in several peripheral tissues as well. CB₂ cannabinoid receptor also known as a peripheral cannabinoid receptor is found in the immune tissues. It is not found in the brain. It is detected at levels of about 10-100 times higher than the CB₁ receptor in the peripheral tissues. High levels of CB₂ receptors found in the immune system suggest that it could have a significant role in immunoregulation by its ligands (Galiegue, 1995). Blood cell subpopulations differ in the degree of CB₂ receptor expression and are ranked in the following order: B cells > natural killer (NK) cells > monocytes > polymorphonuclear (PMN) cells (neutrophils) > CD8⁺ T cells > CD4⁺ T cells (Berdyshev, 2000).

Cannabinoid receptors are also expressed in high density in many areas related to pain. They are found in brain areas involved in descending pain modulation, in the superficial layers of the spinal dorsal horn, in the dorsal root ganglion and on both central and peripheral terminals of primary afferent

neurons. These areas provide peripheral, spinal, and central targets through which cannabinoids modulate pain (Walker and Huang, 2002). The cannabinoid receptors are involved in signal transduction mechanisms that include inhibition of adenylyl cyclase, activation of mitogen-activated protein kinase, and regulation of calcium and potassium channels (Howlett, 2002).

There are indications also that other cannabinoid receptor sub-types may exist that are yet to be identified (Porter and Felder, 2001).

1.5.3 Cannabinoid receptor ligands

Cannabinoids are divided into 5 different chemical classes (see Fig.1.13 for structures).

Classical cannabinoids

The first is the classical cannabinoids. This class consists of natural cannabinoids such as THC_s and some cannabinoids synthesized at Hebrew University in Israel (HU series) such as HU-210 (Guzman *et al.*, 2001) as well as the synthetic selective CB₂ cannabinoid receptor agonist, JWH-133. These are dibenzopyran derivatives (Brown, 1998). The most relevant plant-derived cannabinoids are Δ^9 -THC, Δ^8 -THC (almost as active as Δ^9 -THC but much less abundant in the plant), cannabiol and cannabidiol (both produced in significant amount but are much less potent as cannabimimetic agents). HU-210 is the most potent of these cannabinoids (Guzman *et al.*, 2001). HU-210 has 100-800 times greater potency than Δ^9 -THC (Pertwee, 1995). Because binding values differ due to experimental conditions, data from different laboratories may vary considerably, but the general trend is apparently retained (Howlett *et al.*, 2002). The pharmacophoric elements that appear to be important for cannabimimetic activity are the phenolic hydroxyl group, which can be substituted by an amino group; the alkyl chain, elongation of the chain increases biological activity and; the *para* aliphatic hydroxyl (Goutopoulos and Makriyannis, 2002).

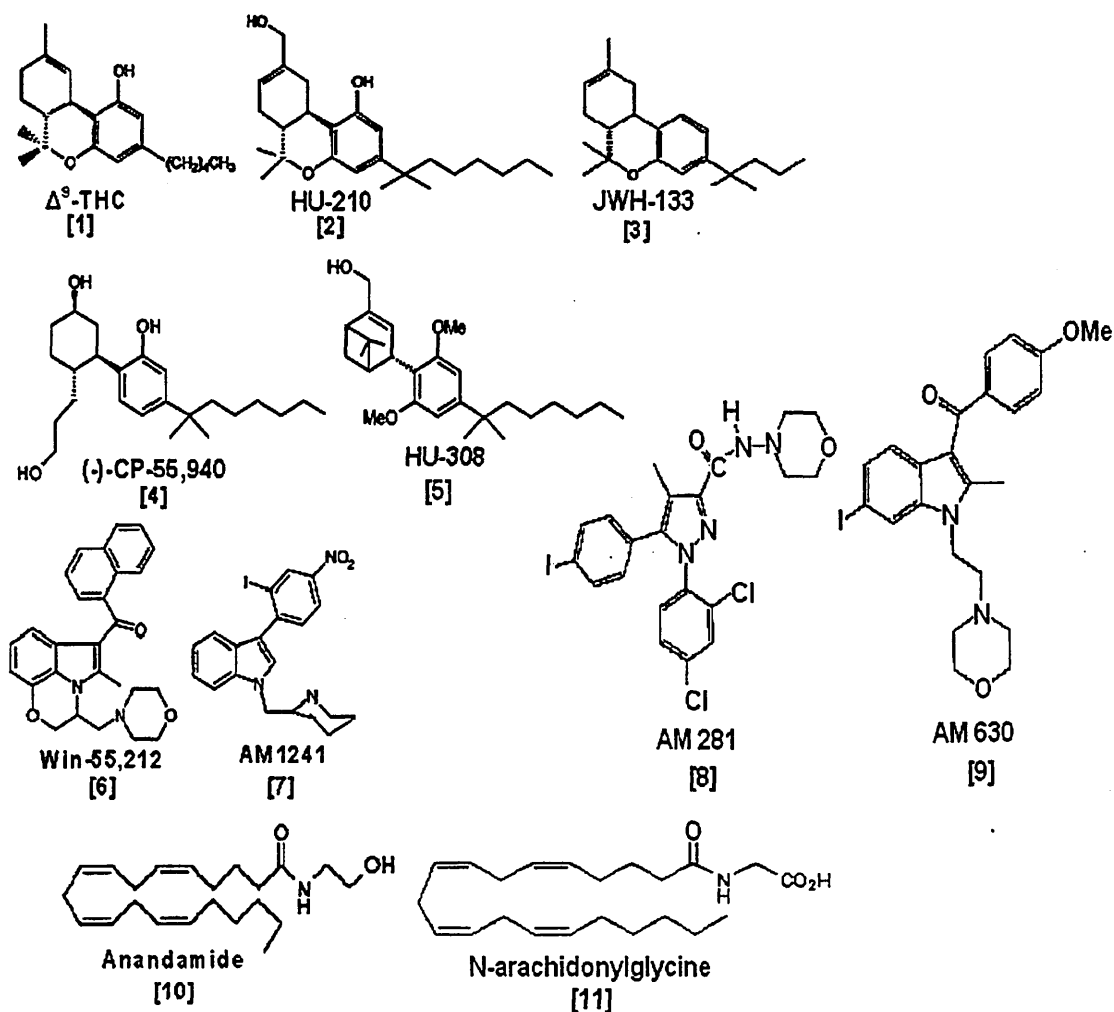


Figure 1.13: Structures of representatives of the cannabinoids [1], [2] and [3] are representatives of the classical cannabinoids; [4] and [5] are non-classical cannabinoids; [6] and [7] are representatives of the aminoalkylindoles; [8] and [9] are CB₁ and CB₂ cannabinoid receptor antagonists (inverse agonists) respectively; and [10] and [11] are representatives of the endocannabinoids (Hanus and Mechoulam, 2005)

Non-classical cannabinoids

The second class is the non-classical cannabinoids. This is comprised of different synthetic bi- and tricyclic analogues of classical cannabinoids lacking the pyran ring. Its main representative is CP-55,940, widely used in its

tritiated form (Brown, 1998). It was the binding assays performed with [³H] CP-55,940 that demonstrated the presence of the cannabinoid receptors (Devane *et al.*, 1988). CP-55,940 is a non selective cannabinoid receptor agonist. CP-55,940 binds to CB₁ and CB₂ receptors with similar affinity and displays high activity *in vivo* as well, being 10 to 50 times more potent than Δ⁹-THC in mouse experiments (Howlett *et al.*, 2002). HU-308 is also a member of this class and it is a CB₂ cannabinoid receptor agonist. The side chain and the phenolic hydroxyl of this class of cannabinoids are important for activity.

Aminoalkylindoles

The third group are the aminoalkylindoles, represented by synthetic cannabinoids, Win-55,212 and AM1241. This class is considerably less lipophilic (Goutopoulos and Makriyannis, 2002). Although quite different in structure to the other cannabinoids, these compounds bind to both cannabinoid receptors (Brown, 1998) and are chemically related to cyclooxygenase inhibitors, although the latter do not bind to the cannabinoid receptors (Guzman *et al.*, 2001).

Endocannabinoids

The fourth class are the endocannabinoids, also known as the eicosanoid group. These are the endogenous cannabinoid receptor ligands, the best known being arachidonylethanolamide (AEA) also known as anandamide, 2-arachidonylglycerol (2-AG) and palmitoylethanolamide (PEA). Anandamide was the first endocannabinoid to be described. It produces similar effects to Δ⁹-THC but is less potent with a short half-life (Devane *et al.*, 1992). It is a ligand for both CB₁ and CB₂ receptors, with less CB₂ than CB₁ efficacy (Pertwee, 1999). Studies by Sugiura and his colleagues (2000) strongly suggest that the CB₂ receptor is originally a 2-AG receptor and that 2-AG is the intrinsic natural ligand for the CB₂ receptor that is abundant in the immune system. PEA has been reported to bind to a yet unidentified CB₂-like receptor (Kumar *et al.*, 2001). In general, endocannabinoids mimic the effects of plant-

derived and synthetic cannabinoids although they are different structurally (Guzman *et al.*, 2001).

Diarylpyrazoles

The last group are the diarylpyrazoles, the cannabinoid receptor antagonists represented by SR141716A and its analogue AM 281; as well as SR144528 and its analogue AM 630, selective CB₁ and CB₂ antagonists (inverse agonists) respectively (Guzman *et al.*, 2001).

1.5.4 The mode of action of cannabinoids

The discovery of cannabinoid receptors and their endogenous ligands suggested the existence of an endogenous cannabinoid system. Apart from receptors and ligands, specific systems for cannabinoid synthesis and degradation have been described. The synthesis of endogenous cannabinoids is believed to be mediated by Ca²⁺-dependent mechanisms involving the activation of a phospholipase D. This enzyme hydrolyses phospholipid precursors releasing anandamide or 2-AG. The degradation system comprises a specific transporter for endocannabinoid uptake and an enzyme that degrades endogenous cannabinoids, fatty acid amide hydrolase (FAAH) (Guzman *et al.*, 2001).

One of the most extensively studied properties of cannabinoid receptors is the ability of the activated receptors to block the accumulation of intracellular cyclic adenosine 3', 5'-monophosphate (cAMP) through inhibition of activation of adenylyl cyclase (AC). The receptors are coupled to AC through the G_{i/o}-proteins. The inhibitory effect of cannabinoid receptor to accumulation of cAMP is abrogated by cell pre-treatment with pertussis toxin which ADP-ribosylates the G-Proteins, thus confirming the G-protein linkage to cannabinoid receptors. The intracellular cAMP level is critical for protein kinase A (PKA)-mediated signalling since cAMP regulates PKA activation. Activated PKA phosphorylates a number of intracellular targets including transcription factors, activator protein-1 (AP-1); the nuclear factor for

immunoglobulin kappa chain in B cells (NF- κ B) and; the nuclear factor of activated T cells (NF-AT) (Berdyshev, 2000). G-proteins also link cannabinoid receptors to mitogen-activated protein (MAP) kinase signalling cascades (Guzman *et al.*, 2001). The regulation of MAP kinases by cannabinoids is complex and may differ depending on cell type, agonist and experimental conditions (Berdyshev, 2000; Bouaboula *et al.*, 1995; Derocq *et al.*, 1998). Cannabinoid receptor-mediated signalling is regulated by the availability of the receptors. Following activation, cannabinoid receptors were shown to undergo phosphorylation and internalisation, which may be followed by recycling back to the membranes (Berdyshev, 2000).

Cannabinoids exhibit a variety of pharmacological properties making them potential candidates for therapeutic development and use in a variety of disorders (Kumar *et al.*, 2001).

1.6 Cannabinoids and the immune system

Effects of cannabinoids on immune function are not obvious; they are fairly complex and appear to be modulatory in nature (Baczynski *et al.*, 1983). The possible existence of a relationship between cannabinoids and immunity was first raised by Juel-Jensen (1972) who reported that marijuana smokers have more frequent recurrences of *Herpes simplex* virus infection. This report gave rise to a large number of further studies on the effect of cannabinoids on immune function (Guzman *et al.*, 2001). Various experimental models have been used employing the drug-abusing human subjects, experimental animals exposed to cannabis smoke or injected with cannabinoids, and *in vitro* models employing immune cell cultures treated with various cannabinoids (Klein *et al.*, 1998). The findings suggested that cannabinoids modulate the function of B and T cells, macrophages, NK cells and mast cells; host resistance to various infectious agents of viral and bacterial origin, especially the secondary immune response; and production and function of cytokines and chemokines (Cabral and Pettit, 1998; Klein *et al.*, 1998).

In most cases, both *in vitro* and *in vivo* studies have shown that cannabinoids have immunosuppressive properties. Contrary to the reports above, cannabinoids were also found to have some immunostimulatory effects; at nanomolar concentrations (10-100nM) (Derocq *et al.*, 2000) and some of the effects were confirmed to be CB₂ receptor-mediated (Jbilo *et al.*, 1999).

This variation in drug effects is a result of different experimental factors such as drug concentration (main factor), timing of drug delivery, and type of cell and cell function being examined (Klein *et al.*, 1998; Guzman *et al.*, 2001).

1.7 Cannabinoids in Pain and inflammation

Link of pain to inflammation

Tissue damage initiated by trauma, infection, allergy or autoimmune reaction may induce production or secretion of inflammatory mediators such as cytokines, prostanoids and neuropeptides (Richardson and Vasko, 2002). These inflammatory mediators produce vascular effects including vasodilation which leads to redness (*rubor*) and heat (*calor*), increase in vascular permeability leading to swelling (oedema) and increase in expression of adhesion molecules leading to recruitment of inflammatory cells (Goldsby *et al.*, 2000). Inflammatory mediators also produce neural effects involving activation of nociceptors, free nerve endings which act as receptors for pain (Costigan and Woolf, 2000). Pain and inflammation are very closely linked. For instance, expression of receptors such as neuropeptide and bradykinin receptors is upregulated during inflammation (Schaible *et al.*, 2002) and this may lead to potentiation of activation of the nociceptors which may result in increased detection of noxious stimuli and the subsequent transmission of encoded information to the brain (nociception) (Kidd and Urban, 2001).

Pain definition

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage, according to the International Association for the Study of Pain (IASP).

Noxious stimuli activate nociceptors, the myelinated A δ fibres which produce fast, sharp pain sensation since they are fast conductors of the pain signals and the unmyelinated C fibres which produce slow, dull pain sensation because they conduct pain signals slowly. These receptors innervate cutaneous as well as deep somatic tissues and have high activation thresholds (Costigan and Woolf, 2000; Stucky *et al.*, 2001). The activated nociceptors, the primary afferent neurons, transduce (detect) noxious stimuli, conduct and transmit the pain signal to the dorsal horn of the spinal cord via the dorsal root ganglion. The neurons and interneurons pass the encoded message via dorsal horn up to the brain where site and type of pain are identified. Thus the pain signal in the brain is processed and induces visceral, emotional and behavioural reactions to the pain (Stucky *et al.*, 2001; and Saladin and Porth, 1998). Repeated exposure of nociceptors to the noxious stimuli leads to increased responsiveness (sensitisation). Associated with this sensitization is a decreased activation threshold such that normally innocuous stimuli may produce pain (allodynia), increased response magnitude to noxious stimuli (hyperalgesia), and increased recruitment of receptive fields (Kidd and Urban, 2001; Winkelstein, 2004).

Effects of cannabinoids in pain states

Cannabinoids have been reported to have anti-nociceptive, analgesic, anti-hyperalgesic, anti-allodynic, anti-inflammatory effects and have potential as therapeutic agents in neuropathic pain (refer to Table 1.3 for a summary of these effects).

These reports of beneficial effects of cannabinoid receptor agonists against the three entities of pain namely: nociceptive, inflammatory and neuropathic; suggest a unique therapeutic role of this group of compounds in pain and inflammation.

Table 1.3: Summary of some of the effects of cannabinoids in pain and inflammation.

Cannabinoid	Receptor type	Effect	Reference
2-Arachidonyl glycerol (2-AG)	CB ₁ /CB ₂	Produced cerebroprotective effects, inhibited vasoconstrictor endothelin (ET-1)	Panikashvili <i>et al.</i> , 2001
AM1241	CB ₂	Inhibited C-fibre mediated nociception Reversed sensory hypersensitivity in neuropathic pain model (topically administered)	Malan <i>et al.</i> , 2003 De Petrocellis <i>et al.</i> , 2000
Anandamide	CB ₁ /CB ₂	Inhibited capsaicin-induced CGRP release	Karst <i>et al.</i> , 2003
Ajulemic acid (CT-3)	CB ₁ /CB ₂	Produced anti-allodynic effects and reduced chronic neuropathic pain Produced analgesia in mouse models of pain, equipotent to morphine but with greater duration of action Inhibited IL-1 production, enhanced T cell apoptosis	Dajani <i>et al.</i> , 1999; Burstein, 2000 Zurier <i>et al.</i> , 2003; Bidinger <i>et al.</i> , 2003
BAY 38-7271	CB ₁ /CB ₂	Exhibited neuroprotective properties in traumatic brain injury TBI	Mauler <i>et al.</i> , 2003
Cannabidiol	CB ₁ /CB ₂	Anti-inflammatory and anti-arthritic	Malfait <i>et al.</i> , 2000
Cannabinol	CB ₁ /CB ₂	Inhibited expression of Th2 cytokines in allergic reactions	Jan <i>et al.</i> , 2003
Dexanabinol (HU-211)		Exhibited antioxidant and anti-inflammatory properties and was cerebroprotective in TBI, prevented secondary degeneration and promoted regeneration	Pop, 2000 Zalish and Lavie, 2003
HU-308	CB ₂	Inhibited C-fibre mediated nociception	Malan <i>et al.</i> , 2003
HU-320	CB ₁ /CB ₂	Inhibited TNF- α and prevented joint damage	Sumariwalla <i>et al.</i> , 2004
Win-55,212-2	CB ₁ /CB ₂	Dose-dependent anti-nociception (topically and intrathecally administered) Reversed hyperalgesia (0.1mg/kg, s.c) by abolishing increases in PGE ₂ , NO and nNOS Reversed development of allodynia and hyperalgesia in neuropathic pain models Inhibited Th1 differentiation and ameliorated MS in mouse model Reduced microglial activity, abrogated MHC class II antigen expression, inhibited proliferation of CD4+ T cells in MS	Drogul <i>et al.</i> , 2003 Costa <i>et al.</i> , 2004 Bridges <i>et al.</i> , 2001 Croxford and Miller, 2003 Arevalo-Martin <i>et al.</i> , 2003
SR141716A (antagonist/inverse agonist)	CB ₁	Enhanced nociception Produced hyperalgesia	De Petrocellis <i>et al.</i> , 2000 Richardson <i>et al.</i> , 1998

1.8 Unwanted effects of *Cannabis* and cannabinoids

Cannabis and cannabinoids can also produce unwanted (adverse) effects, but these have often been overstated according to Vaughan and Christie (2000). The acute toxicity of cannabinoids is very low and no deaths have been directly attributable to their recreational or therapeutic use (Kumar *et al.*, 2001). For a list of some of the adverse effects of cannabis and cannabinoids refer to Table 1.4.

Table 1.4: Unwanted effects of cannabis and some cannabinoids.

Central nervous system	
Effects on mood	Euphoriant effects (↓ anxiety, ↓ alertness, ↓ depression, ↓ tension, ↑ sociability - if taken in friendly surrounding), dysphoric reactions (anxiety, panic, paranoia, transient psychosis), exacerbation of psychotic states e.g. schizophrenia and ↑ risk of suicide
Effects on Perception	↑ sensory perception, distortion of spatial perception and time sense, delusions, hallucinations
Sedative effects	Generalised CNS depression, drowsiness, sleep, additive effect with other CNS depressants
Effects on cognition and psychomotor performance	Slow reaction time, motor in-coordination, fragmented thoughts, mental clouding, subtle impairment of short-term memory and attention, impairs road-driving performance
Tolerance	Develops for many effects including the 'high' and many systemic effects
Dependence and withdrawal syndromes	Potential for development but rare, manifested in restlessness, insomnia, anxiety, aggression, anorexia, muscle tremor and autonomic effects
Cardiovascular system	
Heart rate	Dose-related tachycardia, bradycardia with chronic use
Peripheral circulation	Vasodilation, conjunctival redness, postural hypotension
Eye	↓ intraocular pressure
Immune system	Impaired immunity thus ↓ resistance to infection
Respiratory system	Chronic bronchitis (e.g. coughing, production of sputum and wheezing) and emphysema, ↑ incidence of rare forms of oropharyngeal cancer in chronic young cannabis users
Reproductive system	↓ sperm count and motility, suppression of ovulation, ↑ obstetric risks, low birth weight.

[↑ = increased/enhanced and ↓ = decreased/lowered] (Ashton, 2001; Hall and Solowij, 1998; Kumar *et al.*, 2001; Vaughan and Christie, 2000).

1.9 Cannabinoids in animal models of arthritis

Cannabinoids such as Δ^9 -THC and other natural cannabinoids were shown to possess significant anti-oedema activity in rats (Sofia *et al.*, 1974). Δ^9 -THC was shown to be orally effective not only as an anti-oedema but as analgesic as well and effectively inhibited adjuvant-induced arthritis and suppressed further development of the established disease in rats (Sofia *et al.*, 1973).

Oral administration of ajulemic acid (AjA), a cannabinoid acid devoid of psychoactivity, also reduced joint tissue damage in rats with adjuvant-induced polyarthritis, a model of chronic inflammation and joint tissue injury (Zurier *et al.*, 1998). AjA significantly reduced the severity of adjuvant-induced polyarthritis, histopathologic studies showed attenuation of pannus formation and joint tissue injury (Zurier *et al.*, 1998). In another study, peripheral blood and synovial fluid monocytes (PBM and SFM) were isolated from healthy subjects and patients with inflammatory arthritis, respectively, treated with AjA (0-30 μ M) in vitro, and then stimulated with lipopolysaccharide. Addition of AjA to PBM and SFM in vitro reduced both steady-state levels of IL-1 β mRNA and secretion of IL-1 β in a concentration-dependent manner. Reduction of IL-1 β by AjA may be responsible for the therapeutic effects of AjA in the animal model of arthritis (Zurier *et al.*, 2003).

Growth of synovium as pannus which invades and destroys cartilage and bone in joints of patients with RA depends on activation of several cell types. It has been argued that activated T lymphocytes initiate and govern the process through their failure to undergo apoptotic cell death and thus, induction of T-cell apoptosis may offer a new approach to treatment of diseases such as RA which are characterized by chronic T-cell-driven inflammation (Bidinger *et al.*, 2003). Studies by Bidinger *et al.* (2003) showed that addition of AjA in vitro induces apoptosis of human T lymphocytes. This depletion of T cells by AjA may also be responsible, in part, for AjA suppression of joint tissue injury in the rat model of arthritis above.

Cannabidiol (CBD), the major non-psychoactive component of cannabis has various pharmacological actions of clinical interest and has been shown to be of therapeutic potential as an anti-inflammatory and anti-arthritic in murine collagen-induced arthritis (CIA). It was found that CBD exerted a dose-dependent suppressive action, both on the clinical arthritis and joint damage (Malfait *et al.*, 2000).

1.10 Aim and objectives of the study

Aim

Cannabinoids have been known for centuries to have medicinal properties (section 1.5.1). They have been shown to have immunomodulatory, analgesic and anti-inflammatory effects (sections 1.6 and 1.7). Cannabinoids have also been reported to reduce inflammation and inhibit joint destruction in animal models of arthritis (section 1.9). These reports together suggest that cannabinoids have a potential as anti-arthritic drugs. The mechanisms behind the effects displayed by cannabinoids in animal models of arthritis are not fully elucidated. Therefore the aim of this study was to determine whether cannabinoids have effects on chondrocyte metabolism, which may reduce cartilage breakdown. Since IL-1 has been shown to be a key pro-inflammatory cytokine in arthritis, chondrocytes and cartilage explants were stimulated with IL-1. The effects of cannabinoids on IL-1 α -stimulated cartilage resorption and the production of inflammatory mediators, including NO and eicosanoids were studied and the potential mechanisms for any changes observed. In addition the expression of cannabinoid receptors by bovine chondrocytes was studied.

Objectives

The objectives of the study were to investigate: the toxicity of cannabinoids in chondrocytes; the effect of the drugs on NO, LTB₄ and PGE₂ production; the effect of drugs on protease expression; the effect of drugs on cartilage breakdown; cannabinoid receptor expression in chondrocytes and find out if any of the effects were receptor mediated and; signal transduction pathways involved in chondrocyte metabolism and effects of cannabinoids on these.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Materials

Reagents

Active Motif

(B-1330 Rixensart, Belgium)

FACE™ p38 MAPK kit

Alexis Biochemical, UK (now AXXORA (UK) Ltd)

(Nottingham, UK)

LTB₄ and PGE₂ EIA kits; COX-1 and COX-2 rabbit polyclonal antibodies; mPGES-2 rabbit polyclonal antibody; iNOS polyclonal antibody produced in rabbit

Invitrogen Ltd /Molecular Probes

(Paisley, UK)

Antibiotics: Penicillin/Streptomycin; Collagenase Type I; Dulbecco's modified Eagle's medium (DMEM); Foetal bovine serum (FBS); Fungizone: Amphotericin B; L-Glutamine; Normal goat serum (NGS); Phosphate buffered saline (PBS) without calcium and magnesium; SlowFade anti-fade mounting medium with DAPI; Trypsin (0.25 %); Trypsin-EDTA; Western Blot kit (including pre-cast gels, buffers and membranes); WesternBreeze detection kit; Zymogram gels and buffers.

Santa Cruz Biotechnology

(Santa Cruz, California, USA)

All antibodies used except for those purchased from Alexis and Sigma-Aldrich; Fluorescein isothiocyanate (FITC)-conjugated secondary goat anti-rabbit IgG; Rabbit polyclonal antibodies to CB1 (H-150) and CB2 (H-60); Rabbit polyclonal anti-NF-κB p65; RIPA lysis buffer.

Sigma-Aldrich Company Ltd

(Gillingham, Dorset, UK)

Absolute Ethanol; Acetic acid; Bichinonic acid (BCA) solution; Boric acid; Cannabinoids: Cannabidiol, Cannabinol, Δ 8-THC acid, Win-2 and Win-3; Chloramine T; Citric acid; Crystal violet; Cysteine HCl; p-Dimethylaminobenzaldehyde; 1, 9, Dimethylmethylene Blue; DMSO; EDTA, disodium salt; Glycine; Griess reagent (modified); HCl; 2-isopropanol; LDH assay Kit; Methanol; MTT; n-Propanol; Papain; Perchloric acid; R-250 coomassie brilliant blue; Rat polyclonal anti-cannabinoid receptor -1; Rabbit polyclonal anti-cannabinoid receptor-2; Shark chondroitin sulphate; Sodium acetate trihydrate; Sodium hydroxide; Sodium Nitrite; Trypan Blue.

Tocris Cookson Ltd

(Avonmouth, Bristol, UK)

Cannabinoids: ACEA, AM281, AM630, Anandamide, 2-AG, HU-210, Methanandamide, NADA, N-AG, JWH-133; Glacial acetic acid; NS-398; ODQ; PDTC.

Other sources

Copper sulphate pentahydrate (Cupric sulphate); MK-886

Equipment

Fluorescence microscope coupled with a coolsnap digital camera; Light and Inverted microscopes; UVP BioImaging system with Labworks software (Ultra-Violet Products Ltd); Victor Wallac multi-plate reader; XCell *SureLock*[™] Mini-Cell and XCell II[™] Blot Module Kit (Invitrogen)

2.2 Methods

All the experiments carried out in this study were repeated atleast twice, except where indicated in the figure legend.

2.2.1 Chondrocyte culture

2.2.1.1 Preparation

Bovine ankles were obtained fresh from the abattoir, kept at 4 °C and were used within 24 hrs of slaughter. First the ankles were scrubbed clean under running tap water. Skin around the ankle joint was slit and cut, being careful not to cut the faschia. The joint area was sprayed with 70 % ethanol and wiped with medical tissues. The ends of the joints were wrapped in foil, leaving just the joint exposed. Then the rest of procedures were done in a sterile laminar flow cabinet.

The metacarpophalangeal joint was opened up exposing the articular cartilage by sectioning through the faschia and ligament. The bovine articular cartilage (BAC) was cut from the metacarpophalangeal joint with the aid of a scalpel. The cartilage pieces were washed in phosphate buffered saline (PBS).

The cartilage pieces were sequentially digested enzymatically, first by incubation with 0.25 % trypsin at 37 °C for 30 min, followed by a wash in complete DMEM [supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml) and 10% heat-inactivated FBS (FBS incubated for 30 min in a waterbath set at 56 °C)] to inhibit the activity of the trypsin. Further digestion was accomplished by incubation in sterile filtered type I collagenase (3 mg/ml in complete DMEM) at 37 °C overnight.

The supernatant in the digested tissue was transferred carefully into a sterile tube using a syringe, avoiding the tissue residues. The residues were washed

with PBS and the supernatants were combined and centrifuged at 200 x g (1000 x rpm) for 7 min. The supernatant was discarded and pellets were washed 3-4X with PBS. The cells were then resuspended in complete DMEM and counted. Cell viability was checked using 0.4 % trypan blue solution. Cells were seeded to 2×10^5 cells per cm^2 (Buttle *et al.*, 1997). The chondrocytes were plated and cultured in 24-well plates in a humidified incubator at 37°C in an atmosphere of 5 % CO₂.

2.2.1.2 Culture

The chondrocytes were used as primary cultures. The chondrocytes were incubated in complete DMEM for 5 days and the medium was changed. After 5-10 days, the chondrocytes were incubated with or without cannabinoids \pm IL-1 α (100 U/ml \equiv 1 ng/ml \equiv 0.06 nM) for 48 hrs in DMEM with the supplements except FBS at 37°C in an atmosphere of 5 % CO₂.

2.2.2 Cartilage explant culture

2.2.2.1 Preparation

Bovine noses were obtained as above (section 2.2.1). Bovine nasal cartilage (BNC) (Buttle *et al.*, 1993) was prepared by dissecting the septum from the nasal bony hard palette. The septum was sprayed with ethanol and transferred to a sterile laminar flow cabinet and put in a Petri dish. The septum was cleaned carefully by removing the mucous membrane and washing in PBS a few times. Slices (approximately 3 mm) were cut using a sterilized Mandolin. BNC discs (3mm in diameter) were prepared using the sterile leather-belt punch. The discs were washed 3X in PBS.

2.2.2.2 Culture

The BNC explants were incubated in complete DMEM for at least 24 hrs. Before the experiments were set up, the medium was changed to DMEM

without FBS. Discs were plated out in 96-well plate (1 disc/well/200 μ l medium) or 24-well plate (1 or 3 discs/well/600 or 1000 μ l medium). The BNC explants were incubated with or without cannabinoids \pm IL-1 α (500 U/ml \equiv 5 ng/ml \equiv 0.3 nM) (Buttle *et al.*, 1997) for 4 days or 21-35 days at 37 °C in an atmosphere of 5 % CO₂. The conditioned medium was removed and stored at -20 °C until assay and the explants were also stored the same way prior to digestion.

2.2.3 Cytotoxicity assays

2.2.3.1 MTT assay

This is colorimetric assay that is used as a measure viable cell number as the formation of the coloured product depends on cell metabolic activity. This is used for quantification of cell proliferation as well as cell cytotoxicity.

Principle

MTT is water soluble producing a yellowish solution. This is converted to insoluble blue formazan salt by cleavage of the tetrazolium ring by dehydrogenases of mitochondria of living cells. Acid-isopropanol or other solvents is added to the wells to dissolve the formazan crystals. Absorbance is read at 570 nm using the multi-plate reader. The absorbance is determined as measure of cell number (Mossman, 1983).

Procedure

Filter sterilised MTT (5 mg/ml in PBS), 10 μ l per 100 μ l medium, was added to all the wells of the assay, and the plate was incubated at 37 °C for 4 hrs. The medium was removed and acid-isopropanol was added, 100 μ l per well, to dissolve the blue formazan crystals in the cell layer. Dissolution took about 30 min and absorbance was read at 570 nm.

2.2.3.2 Crystal violet assay

Principle

The cells, following culture were fixed and stained with crystal violet. The stain bound to the DNA of the cells. Excess stain was washed away with water. Acetic acid was then used to dissolve the bound stain. Absorbance of the stain was measured and was related to cell number.

Procedure

The assay was carried modified from (Kaeffer *et al.*, 1997). Culture medium was removed and cells were washed in 200 μ l of PBS. Cells were fixed with 200 μ l of methanol for 15 min. Methanol was removed and the plates were air dried in a fume cupboard. The plates were stained with 0.1 % crystal violet (in 200 mM boric acid), 200 μ l/ well for 20 min. The plates were washed with distilled water and the stained cell layer was solubilised in 50 μ l of 10 % glacial acetic acid. The plate was incubated at 37 °C for 30 min to achieve dissolution. Absorbance was read at 550 nm.

2.2.3.3 LDH release assay

LDH assay was used as a means of measuring membrane integrity, determined by measuring lactate dehydrogenase (LDH) in the extracellular medium. This enzyme is normally present in the cytosol, and cannot be measured extracellularly unless cell damage has occurred.

Principle

LDH activity is determined in an enzymatic test. In the first step, NAD⁺ is reduced to NADH/H⁺ by the LDH-catalyzed conversion of lactate to pyruvate.

In the second step, the H/H⁺ are transferred from NADH/H⁺ to a tetrazolium salt which is reduced to formazan. The formazan dye is water soluble and shows a broad absorption maximum at about 500 nm, whereas the tetrazolium salt shows no significant absorption at this wavelength. Absorbance indicates the degree of cytotoxicity. Amount of LDH activity can be used as an indicator of relative cell viability as well as a function of membrane integrity (Legrand *et al.*, 1992).

Procedure

The assay was performed according to the manufacturer's instructions. Briefly, LDH assay enzyme preparation was reconstituted and the assay mixture was prepared containing substrate, enzyme and dye according to the manufacturer's (Sigma-Aldrich) instructions. 100 µl of conditioned medium from each well was transferred into a clean plate. Then 50 µl of assay mixture was added to the conditioned medium in the wells. The plate was covered with foil and incubated at room temperature for 20-30 min. The reaction was stopped by adding 1/10 of total volume (in the well) of 1 M HCl to each well. Absorbance was read at 490 nm.

2.2.4 NO production assay

NO gas has an extremely short half-life (< 10 s), which makes it difficult to detect and study directly. However, because NO is metabolized to nitrite and nitrate, quantification of these stable anions can be used to indirectly determine the amount of NO originally present. In this study NO was measured as one of its stable metabolites, nitrite.

Principle

Nitrite reacts with sulfanilamide releasing water to produce an intermediate product which then reacts with N-(1-Naphthyl) ethylenediamine to form a coloured Azo-product that absorbs light at wavelength approximately around 550 nm (Fig. 2.1).

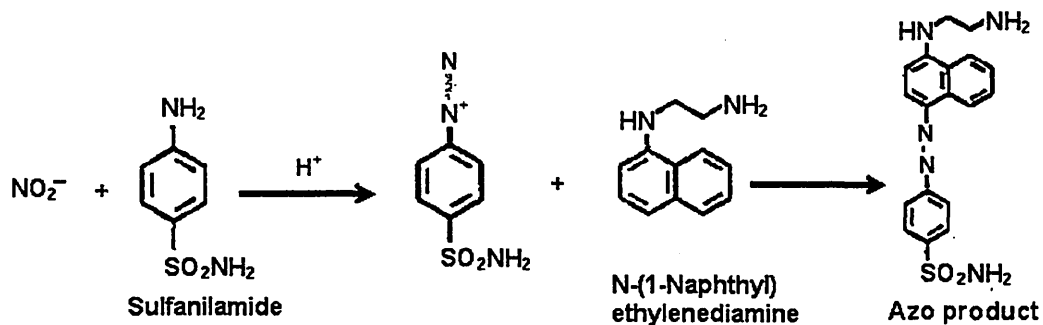


Figure 2.1: Chemical reactions in a Nitrite Assay. Two step conversion of nitrite to a colored azo-product by chemically reacting with sulfanilamide and N-(1-naphthyl) ethylenediamine.

Procedure

NO production was measured as nitrite using the Griess reagent (Green *et al.* 1982). Equal amounts (100 μl) of each of Griess reagent (Sigma-Aldrich) and conditioned medium or standard (0-100 μM) or blank (culture medium alone) were added to a 96-well plate, adding Griess reagent last. The plate was allowed to stand at room temperature for 10 min for colour to develop. Absorbance was read at 550 nm. Nitrite concentration in the sample was calculated from a standard curve, 0 -100 μM sodium nitrite (Fig. 2.2).

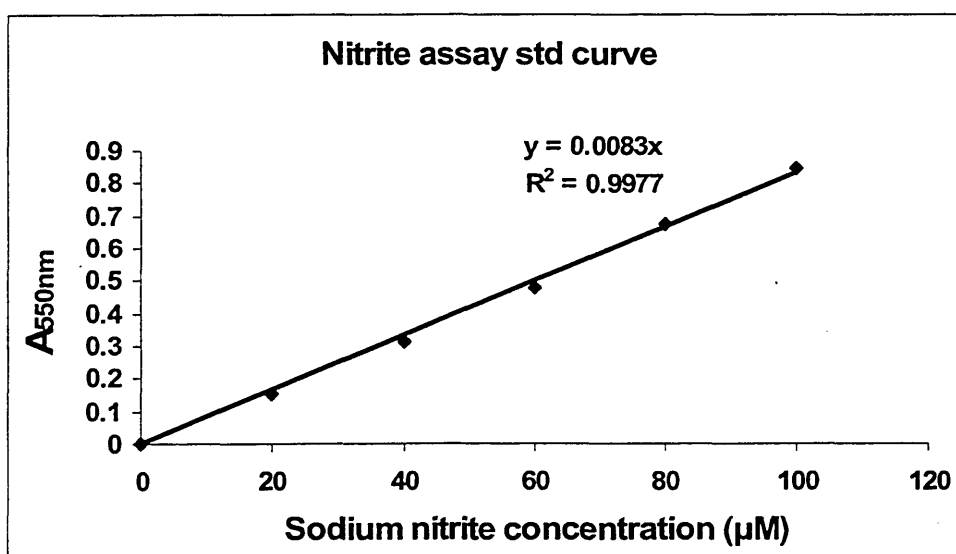


Figure 2.2: A typical nitrite assay standard curve

2.2.5 PGE₂ and LTB₄ production assays

PGE₂ and LTB₄ production was measured using Enzyme ImmunoAssay (EIA) kits, following the instructions from the manufacturers (Alexis Biochemical).

Contents of the Kits

Table 2.1: EIA kit contents

Number	Item	96 wells Quantity/ Size
1	PGE ₂ monoclonal antibody/ LTB ₄ antiserum	1 vial/ 100 determinations (dtn)
2	PGE ₂ / LTB ₄ EIA AChE tracer	1 vial/ 100 dtn
3	PGE ₂ / LTB ₄ EIA standard	1 vial/ 1 each
4	EIA buffer concentrate	2 vial/ 10 ml
5	Wash buffer concentrate	1 vial/ 5 ml
5a	Tween 20	1 vial/ 3 ml
6	Goat anti-mouse IgG coated plate	1 plate/ 1 each
7	Plate cover	1 cover/ 1 each
8	Ellman's reagent	3 vials/ 100 dtn
14	EIA tracer dye	1 vial/ 1 each
15	EIA antiserum dye	1 vial/ 1 each

Principle of the Assay

The assay is based on the competition between PGE₂/ LTB₄ and a PGE₂/ LTB₄-acetylcholinesterase (AChE) conjugate (tracer) for a limited amount of PGE₂ monoclonal antibody/ LTB₄ antiserum. The concentration of the tracer is held constant while concentration of PGE₂/ LTB₄ in the samples varies; therefore the amount of tracer that is able to bind to the antibody will be inversely proportional to the concentration of PGE₂/ LTB₄ in the wells.

This antibody- PGE₂/ LTB₄ complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the wells. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to AChE) is added to the wells. The product of this enzymatic reaction has a distinct yellow colour which absorbs strongly at 412 nm. The

intensity of this colour, determined spectrophotometrically, is proportional to the amount of tracer bound to the wells, which is inversely proportional to the amount of free PGE₂/ LTB₄ present in the wells during the incubation. For a summary of the assay process refer to Figure 2.3.

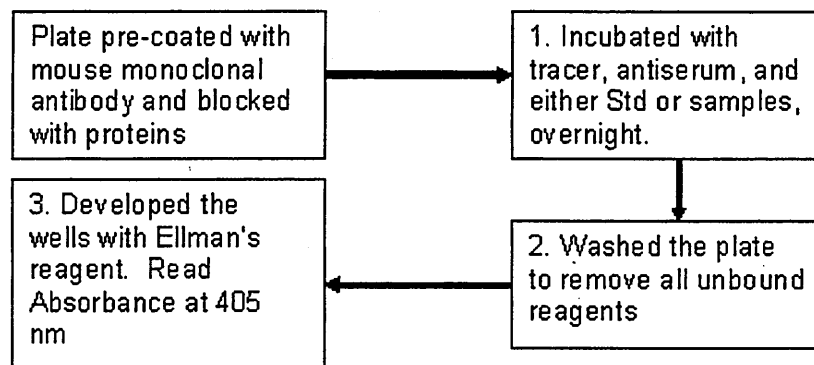


Figure 2.3: Schematic drawing of the EIA process.

Biochemistry of the EIAs

AChE is a stable enzyme that hydrolyses acetylthiocholine. In the enzyme immunoassays, a molecule of the analyte (in this case, PGE₂ or LTB₄) covalently bound to a molecule of AChE serves as the tracer. The quantification of the tracer is achieved by measuring its AChE activity with Ellman's reagent. This reagent consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine. The non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm. See Figure 2.4.

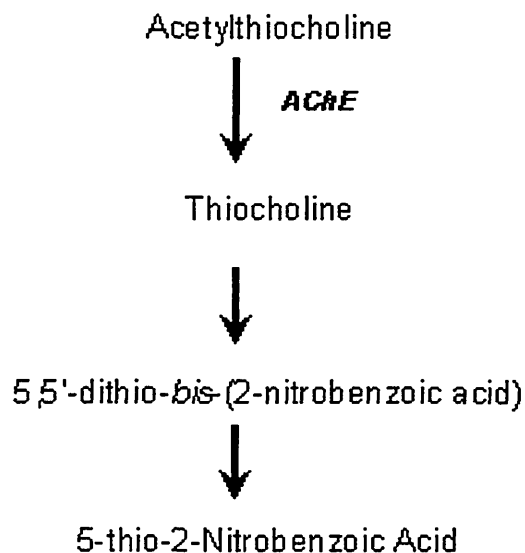


Figure 2.4: The Biochemical reactions in the EIAs

Pre-assay preparations

EIA buffer was prepared by diluting the contents of one vial of EIA buffer concentrate with 90 ml deionised and sterilised water. Wash buffer was prepared by diluting the vial (5 ml) of wash buffer concentrate with deionised water to a total volume of 2 L and 1 ml Tween 20 was added to it. To prepare 1 L of wash buffer, 2.5 ml of wash buffer concentrate was diluted to 1 L, and 0.5 ml Tween 20 was added. Conditioned media was used immediately at the end of the cell culture. No dilution was done.

Preparation of assay-specific reagents

The PGE₂ standard was reconstituted with 1 ml of EIA buffer making the stock solution (10 ng/ml). The standard curve was diluted with culture media because the samples were not diluted. Serial dilutions were prepared for the standard curve. Eppendorf tubes were set aside, numbered 1 to 8. 900 µl of culture medium was aliquoted to tube 1 and 500 µl of culture medium was aliquoted to tubes 2 to 8. 100 µl of stock solution (10 ng/ml) was transferred to the tube 1 and mixed thoroughly. The standard was then serially diluted by transferring 500 µl from tube 1 to tube 2, and from tube 2 to tube 3 and so on,

mixing thoroughly prior to each transfer. The diluted standards were not stored for more than 24 hrs.

LTB4 standard preparation started with equilibration of the pipette tip used in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, 100 pi of LTB4 standard was transferred into a clean Eppendorf tube, and 900 pi was then added to this tube using deionised, sterilised water to make a stock solution (5 ng/ml). The standard curve was diluted with culture media because the samples were not diluted. Serial dilutions were prepared for the standard curve as for the PGE2 standards (Fig. 2.5).

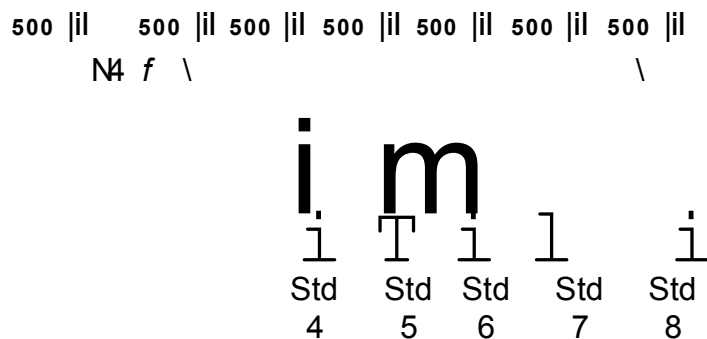


Figure 2.5: Standards serial dilution

PGE2LTB4 AChE Tracer (1 vial) was reconstituted with 6 ml EIA buffer. PGE2LTB4 antiserum was also reconstituted with 6 ml EIA buffer.

Assay procedure

Each plate or set of strips contained a minimum of 2 blanks (Blk), 2 non-specific binding (NSB) wells, 2 maximum binding (B0), and an 8 point Std curve run in duplicate. 50 pi of culture medium was added to NSB and B0 wells and 50 pi EIA buffer to NSB wells. 50 pi was added to the Std wells, starting with the lowest concentration continuing with this procedure until all the Stds were aliquoted. 50 pi of sample was added per sample well. Each sample was assayed at least in duplicate. PGE2 LTB4 AChE tracer was

added (50 μ l) to each well except the Total Activity (TA) and the Blk wells. PGE₂ monoclonal antibody/ LTB₄ antiserum was added (50 μ l) to each well except the TA, NSB and Blk wells. The plate was covered with plastic film and incubated overnight at 4°C.

For the development of the plate, Ellman's reagent was reconstituted with 20 ml of deionised and sterilised water and was used the same day since it is unstable. It was also protected from light by covering the tube containing the reagent with aluminium foil. The wells were emptied and rinsed 5X with wash buffer (1X). 200 μ l of Ellman's reagent was added to each of the wells and 5 μ l of tracer was added to the TA wells. The plate was covered with plastic film and protected from light by covering with aluminium foil. The plate was placed on a shaker for optimal development. The assays developed (i.e. B₀ wells read a minimum of 0.3 at 405 nm, with the Blk subtracted) in 90-120 minutes. The plate was read at a 405 nm, when the B₀ wells were in the range 0.3-0.8 (Blk subtracted). If the absorbance of the wells exceeded 1.5, the plates were washed and fresh Ellman's reagent was added and plate was developed again.

Calculation of the results

The absorbance readings from the NSB wells were averaged. The absorbance readings from the B₀ wells were also averaged. The NSB average was subtracted from the B₀ average. This is the corrected B₀ or corrected maximum binding. The % B/B₀ (% sample or Std bound/maximum bound) for the remaining wells was calculated. To do this, the average NSB absorbance was subtracted from the Std 1 absorbance and divided by the corrected B₀ (from step 3). This was then multiplied by 100 to obtain % B/B₀. The calculation was repeated for Std 2-8 and all sample wells. The standard curve was prepared by plotting % B/B₀ for Std 1-8 vs PGE₂/ LTB₄ concentration (in pg/ml) on semi-log scale. Actual PGE₂/ LTB₄ concentration was calculated using the equation of the plot (Fig. 2.6). The detection limit (80% B/B₀) for PGE₂ EIA assays was generally approximately 0.05 pg/ml, and for LTB₄ EIA assays was in general around 0.002 pg/ml.

STD Curve for PGE2Assay

$$y = -5.8914\ln(x) + 36.164$$

$$R^2 = 0.974$$

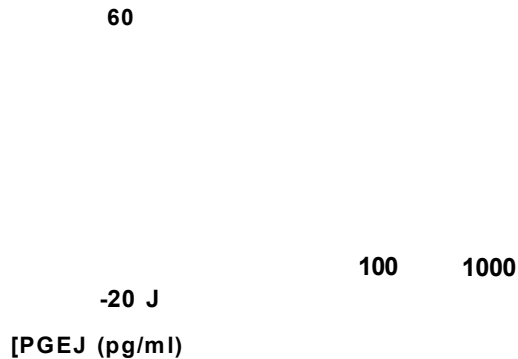


Figure 2.6: A typical standard curve for the PGE2 assay

2.2.6 Cartilage resorption assays

2.2.6.1 Collagen release assay

Bovine nasal cartilage explants when incubated in the presence of IL-1 α (0.3 nM) were stimulated to undergo extracellular matrix resorption and this included breakdown and release of collagen later in the resorption process. Collagen breakdown was measured as hydroxyproline (Hpro) released in the conditioned media using the Hpro assay described by Reddy and Enwemeka (1996).

Principle of the assay

The method was based on alkaline hydrolysis of samples (conditioned media or residual cartilage digests) and subsequent quantification of free Hpro in the hydrolysates. It involved 3 main steps: (a) hydrolysis to free Hpro using NaOH (2 M final concentration), (b) oxidation of Hpro by chloramine T reagent, and (c) chromophore development by the Ehrlich's reagent.

Assay reagents

Acetate-citrate buffer (pH 6.5)

Sodium acetate trihydrate (120 g), citric acid (46 g), acetic acid (12 ml) and NaOH (34 g) were dissolved in 1 L in distilled water with pH adjusted accordingly to 6.5.

Chloramine T reagent (0.056 M)

Chloramine T (1.27 g) was dissolved in 20 ml 50 % n-propanol and brought to 100 ml with acetate-citrate buffer.

Ehrlich's reagent (1 M)

p-Dimethylaminobenzaldehyde (15 g) was dissolved in n-propanol/perchloric acid (2:1, v/v) and brought to 100 ml. Since this reagent is not stable, it was prepared freshly before each assay.

The residual cartilage was digested using papain solution containing 25 mg/ml papain in 0.1 M phosphate buffer (pH 6.5); 50 mM EDTA disodium salt and 50 mM cysteine HCl. Sodium hydroxide (10 M) in distilled water was prepared as a stock solution. L-hydroxyproline was used for standard curve. A solution containing 1 mg/ml of Hpro in distilled water was prepared as a stock solution.

Assay procedure

For residual cartilage digestion, cartilage from each well was digested in an Eppendorf tube with a total volume of 700 µl containing: 490 µl phosphate buffer, 70 µl EDTA disodium salt, 70 µl cysteine HCl and 70 µl papain. The papain used was fairly insoluble therefore the digestion buffer mix was filtered prior to addition to the tubes containing the discs. The capped tubes were incubated at 65 °C in a water bath for 2 hrs to digest the discs.

Hydroxyproline content was determined in the conditioned media as well as in digests following the procedure below:

10 µl of standard (0-50 µg/ml) and samples (conditioned media and digests) were mixed with NaOH (2 M final concentration) in a total volume of 50 µl. The samples were autoclaved at 120 °C for 20 min (for hydrolysis) in O-ring screw-capped polypropylene (PP) tubes (2 ml). 450 µl of chloramine T was added to the hydrolysates and standards were mixed gently followed by incubation at room temperature for 25 min to allow oxidation to take place. 500 µl of Ehrlich's reagent was added to each sample and standard and mixed gently. Chromophore was allowed to develop by incubating at 65 °C for 20 min. Absorbance was read at 550 nm in 96-well plate using a Victor-Wallac plate reader. The equations obtained from the prepared standard curves (see Fig. 2.7) were used to determine the actual concentration of the released hydroxyproline, taking into consideration the dilution factors. The results were expressed as a percent cumulative hydroxyproline released (as a percentage of total hydroxyproline in the explants per well).

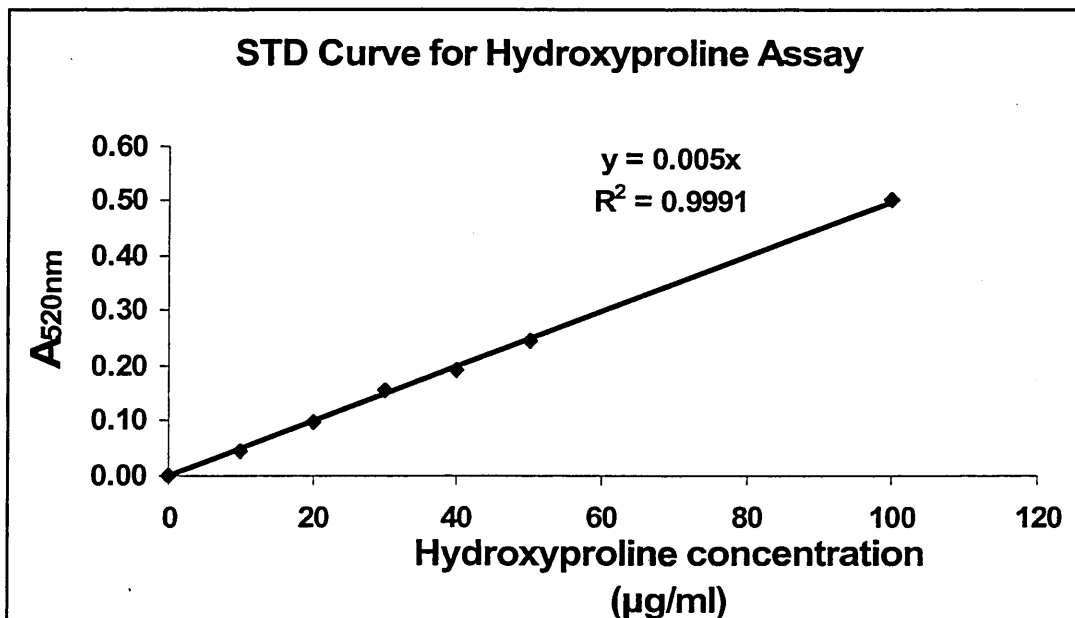


Figure 2.7: A typical hydroxyproline assay standard curve.

2.2.6.2 Cartilage Oligomeric Matrix Protein (COMP) release assay

This assay was used to measure the release of COMP fragments into the conditioned media, as one of the markers of extracellular matrix degradation. COMP is one of the biomarkers of cartilage resorption (Neidhart *et al.*, 1997). It can also be used prognostically for cartilage destruction in inflammatory arthropathies such as RA and OA (Forslind *et al.*, 1992) since quantitative relation has been found between COMP concentration in serum and the degree of cartilage destruction. This animal COMP ELISA from AnaMar Medical (Sweden) provides a method for the determination of COMP in animal serum including that of bovine. The assay was carried out following the manufacturer's instructions. Conditioned medium was used in place of serum.

Assay kit reagents

(See Table 2.2 below)

Table 2.2: COMP ELISA kit reagents

Item	Quantity	Preparation
COMP coated plate (COMP of bovine origin)	1 plate	Ready for use, stored at 4° C
Calibrators (rat origin) (0.055 U/L-0.9 U/L)	5 vials (0.5 ml)	Ready for use
Sample buffer	1 vial (7.5 ml)	Ready for use
Polyclonal antibody	1 vial (7 ml)	Ready for use
Enzyme conjugate 11X peroxidase conjugated antibody (donkey origin)	1 vial (1.2 ml)	Concentrate, prepared by diluting 11X in conjugate buffer
Conjugate buffer	1 vial (12 ml)	Ready for use
Stop solution 0.5M H ₂ SO ₄	1 vial (7 ml)	Ready for use
Washing tablet	2 tablets	Dissolved in 500 ml deionised water
Enzyme substrate (TMB)	2 vials (6 ml)	Ready for use

Principle of the procedure

In the competitive COMP ELISA, the wells of the 96-well plate were provided coated with bovine COMP. Sera from rats were used as calibrators. A

polyclonal antiserum from rats directed against COMP was used as the primary antibody. This antibody was incubated together with samples and calibrators directly in the wells, so the antibody would competitively bind to either the COMP coated to the well or to one added as sample or calibrator. After washing, donkey anti-rat peroxidase conjugate was added to the wells so it could bind to primary antibody. The peroxidase enzyme usually catalyses the oxidation of a chromogen that can be measured following termination of the reaction. A chromogen widely used for this purpose is 3,3',5,5'-tetramethylbenzidine (TMB). The two electron oxidation of tetramethylbenzidine yields a component with an absorbance maximum at 450 nm. If the enzyme reaction is terminated by lowering of the pH (less than 1.0), an additional increase of the absorbance at 450 nm is observed (Bally and Gribnau, 1989). Therefore after washing, the enzyme substrate TMB was added. The product of this enzymatic reaction changed colour to yellow when the stop solution (sulphuric acid) was added. The absorbance of the colour developed was read at 450 nm. This response was inversely proportional to the concentration of COMP in the sample.

Assay procedure

The samples (conditioned media) were diluted 1/10 in sample buffer. Sample buffer (blank) and Std 0.9 U/ml – 0.055U/ml in 50 µl aliquots were added to the Std wells. 50 µl of conditioned medium were added to the sample wells followed by addition of 50 µl polyclonal antibody to all the wells. The plate was sealed and incubated on a shaker for 2 hrs at room temperature. After the incubation, the plate was washed 6 times. After the final wash, the plate was inverted and tapped firmly against a paper towel. 50 µl of conjugate was added to each well. The plate was sealed and incubated on a shaker for 1 hr at room temperature after which the plate was washed 6 times. After the final wash, the plate was inverted and tapped firmly against the paper towel. 100 µl of enzyme substrate was added to each well. The plate was incubated for 15 min at room temperature. 50 µl stop solution was added and the plate was placed on a shaker for approximately 5 seconds to ensure mixing of substrate and stop solution. The absorbance was then read at 450 nm. The calibrator

curve was prepared by plotting absorbance versus calibrator concentration on a semi-log scale (Fig. 2.8). The equation from this plot was used to work out the actual COMP concentration in the samples, multiplying the result by dilution factor (x10).

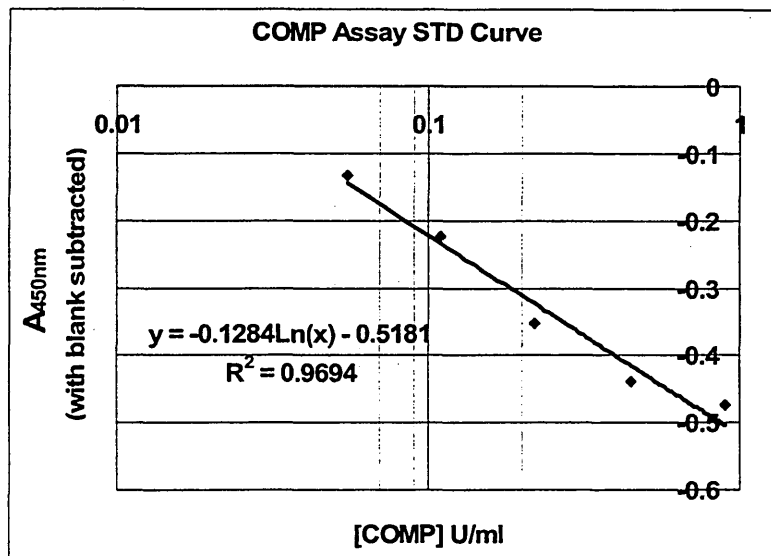


Figure 2.8: A typical COMP assay standard curve

2.2.6.3 Proteoglycan release assay

Bovine nasal cartilage explants when incubated in the presence of IL-1 α (0.3 nM) underwent extracellular matrix resorption (Buttle *et al.*, 1997) that included the breakdown and release of proteoglycan earlier in the catabolic process. Proteoglycan breakdown was measured as release of sulphated glycosaminoglycans (sGAGs), such as chondroitin sulphate, using the dimethylmethylene blue assay (Farndale *et al.* 1986).

Assay principle

1,9-dimethylmethylene blue binds with the sGAGs causing an increase in absorbance at 520 nm dependent on the amount of sGAGs present. This was used to quantify cartilage resorption in terms of proteoglycan breakdown.

Assay reagents

Dimethylmethylene blue solution was prepared containing 16 mg 1,9-dimethylmethylene blue in 1 L of Glycine buffer (3.04 g glycine, 2.37 g NaCl and 95 ml 0.1 M HCl in 1 L; adjusted to pH 3.0 using HCl or NaOH). The absorbance of the solution was checked at 520nm and was expected to be around 0.31. The solution was stored in a brown bottle (to protect from light) at room temperature and was stable for several months. This solution was used to determine the amount of sGAGs measured as chondroitin sulphate in conditioned media and cartilage digests.

The residual cartilage was digested using papain solution containing 25 mg/ml papain in 0.1 M phosphate buffer (pH 6.5), 50 mM EDTA disodium salt and 50 mM cysteine HCl.

Chondroitin sulphate stock solution, 4 mg/ml chondroitin sulphate (shark or bovine trachea) (Sigma-Aldrich) was made up in either DMEM or phosphate buffer (pH 6.5) depending on whether sGAGs were being measured in conditioned media or digests. Standard was kept at -20 °C in aliquots.

Procedure

Residual cartilage digestion was carried out as explained under section 2.2.6.1. Standard stock solution of 4 mg/ml chondroitin sulphate was diluted to 40 µg/ml working solution and standard curve was constructed using 0-40 µg/ml chondroitin sulphate. The test samples (conditioned media and digests) were diluted 100X.

Aliquots, 40 µl, of the standard or test samples were added to wells of a 96-well plate. Dimethylmethylene blue solution, 250 µl, was added to the wells and absorbance was read immediately at 520 nm using Victor-Wallac plate reader spectrophotometer.

The equations obtained from the prepared standard curves (Fig. 2.9) were used to determine the actual concentration of the released sGAGs, taking into consideration the dilution factors. The results were expressed as a percent of cumulative sGAGs released (as a percentage of total sGAGs in the explants per well).

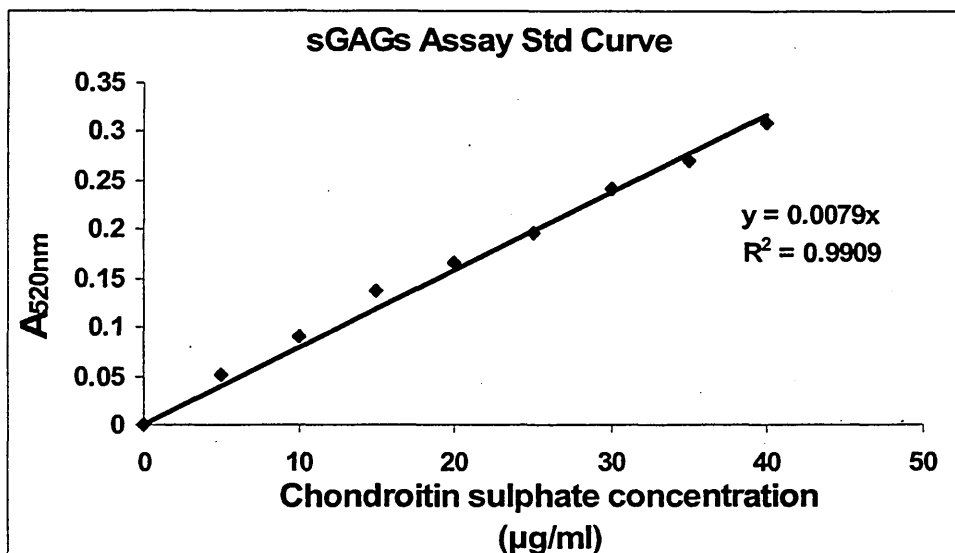


Figure 2.9: A typical proteoglycan release assay standard curve.

2.2.7 Immunoblot analysis

Principle

Immunoblot analysis also known as western blotting is a method for the identification of a specific protein that involves: sample protein separation by size using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); transfer of separated proteins to a membrane; and specific identification by labeled antibodies. The first step in immunoblot analysis was gel electrophoresis, in which the proteins of the sample were separated according to size on a gel using SDS-PAGE. SDS coats the protein and gives it an overall negative charge such that when a current is applied, the proteins migrate towards the positive electrode, being separated in the process according to the molecular weight. The proteins in the gel were then

transferred onto a membrane made of polyvinylidene difluoride (PVDF), by applying an electric current (electroblotting). The membrane binds proteins non-specifically. This process is necessary in order to expose the proteins for detection and the membrane is used because it is more manageable than a gel. The membrane was then blocked, in order to prevent non-specific protein interactions between the membrane and the antibodies used to detect the protein. Without the blocking, the antibodies would bind to the membrane instead of binding to the protein of interest only. The membrane was incubated in primary antibody. The primary antibody should recognise only the protein of interest on the membrane. After washing the membrane to remove unbound primary antibody, the membrane was incubated with a secondary antibody, goat anti-rabbit antibody conjugated to alkaline phosphatase. The unbound secondary antibodies were washed away, and the membrane incubated with the enzyme substrate so that the positions of membrane-bound secondary antibodies could be visualised in this case as purple band(s). In principle, the protein bands on the membrane that are stained represent the protein(s) of interest. The size of the protein was confirmed using the protein markers run on the same gel as the samples.

Whole cell lysate preparation

Cells were maintained as primary cultures and stimulated using IL-1 α (0.06nM) following 30 min pre-treatment with Win-55,212-2 and incubated for 48 hrs. The cells were washed in PBS and kept on ice. Complete lysis buffer was prepared by mixing radioimmuno precipitation assay (RIPA) lysis buffer (5ml), phenylmethylsulfonyl fluoride (PMSF) (50 μ l), sodium orthovanadate (50 μ l) and protease inhibitor cocktail (100 μ l) according to the manufacturer's instruction (Santa Cruz). The cells were incubated with the complete lysis buffer for 1 hr on ice. The whole cell lysates were centrifuged at 10,000xg for 10 min at 4 °C. The supernatant was stored at -20 °C until further analysis.

Protein concentration determination

This was done to determine protein concentration of samples. The reagent was prepared by mixing: Bichinichonic acid solution- BCA reagent (Sigma); Copper (II) sulphate pentahydrate solution (4 % v/v), BSA (1mg/ml) in lysis buffer. The working reagent was prepared by adding 1 ml of copper (II) sulphate solution to 49 ml of BCA reagent. BSA was used as a standard and its stock solution was prepared at 1mg/ml. Standard curve was prepared in the range of 0-25µg/ 25 µl.

25 µl of standards or sample were added to a 96 well plate. 200 µl of working reagent was added to the wells and the plate was placed on a shaker for 1 min. Thereafter the plate was incubated for 30 min at 37 °C. Absorbance was read at 570 nm. Protein concentration for the samples was in the range of 9-14 µg/ 25µl.

2.2.7.1 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

Running buffer

NUPAGE® Novex Bis-Tris (3-(N-morpholino) propanesulfonic acid (MOPS)) running buffer was used. 500 µl of antioxidant was added to 200 ml of the 1X running buffer and this was used to fill the upper chamber of the XCell *SureLock*® Mini-Cell.

Sample preparation

5 µl lithium dodecyl sulphate (LDS) sample buffer and 2 µl of reducing agent (dithiothreitol (DTT)) were added to samples. Samples were heated at 70 °C for 10 min, according to the manufacturer's instructions (Invitrogen).

Gel cassette's preparation and electrophoresis

1.0 mm and 1.5 mm thick pre-cast 4-12 % NuPAGE® Bis-Tris mini-gels were used. Gel cassettes were removed from their pockets, rinsed with deionised

water. The slot at the bottom was stripped open, on one side of the gel. The comb was removed and gel wells were washed 3X and filled with the running buffer containing antioxidant. The gel cassette was loaded into the electrophoresis module of XCell SureLock® Mini-Cell, with the slot facing out and wells facing toward the upper chamber core. The upper chamber was filled with approximately 200 ml of the running buffer containing antioxidant, covering the wells of the gel. SeeBlue plus2 pre-stained protein standards were used for easy and clear band identification. Up to 5µl of the marker as well as up to 35 µl of samples were loaded into the wells of the gel. The lower chamber of the electrophoresis module was filled with approximately 600 ml of the running buffer without the antioxidant. 2 gels were normally run simultaneously. The electrophoresis running conditions were set at 200V, 125mA, 35 W for 1 hr in MOPS buffer.

2.2.7.2 Protein transfer

The electrophoresed proteins on NuPAGE® gels were electroblotted onto Invitrolon® PVDF membranes using the XCell II™ Blot Module. Invitrolon™ PVDF is a 0.45 µm membrane which is particularly suitable for high sensitivity and low background immunoblotting. NuPAGE® transfer buffer (1X) was prepared by adding 50 ml 20X NuPAGE® transfer buffer and 200 ml methanol to 750 ml deionised water. 1ml NuPAGE® antioxidant was added to the buffer for the reduced samples.

Blotting pads were soaked in the transfer buffer and bubbles in the pads were removed. The PVDF membrane was washed in methanol for 30 sec, rinsed with deionised water. The membranes as well as filter papers were equilibrated in the transfer buffer for 5 min. The gels were removed from the cassettes for transfer by following manufacturer's instructions (Invitrogen). Briefly, the cassettes were opened using a gel knife. The gel rested on one of the plates of the cassette. Pre-soaked filter paper was placed on the gel and bubbles were removed by rolling a pipette on the filter paper. The filter paper was soaked with the transfer buffer to prevent the gel from drying. The wells part of the gel was removed using the gel knife. The assembly was turned

upside-down over a clean flat surface covered with parafilm. The gel was allowed to free fall from the cassette plate, the foot was cut off. Pre-soaked transfer membrane was placed on the gel and air bubbles were removed. Another pre-soaked filter paper was placed on top of the membrane and any air bubbles were removed. The filter paper was wet with transfer buffer. Two gel/membrane sandwiches were prepared for each transfer.

Two pre-soaked blotting pads were placed on the cathode (-) core of the blot module. This was followed by the first gel/membrane sandwich, with the gel closest to the cathode core. Another blotting pad was placed on the first assembly followed by the second gel/membrane assembly, again with the gel closest to the cathode core. Two more pre-soaked blotting pads were placed on top of the second assembly, followed by the anode (+) core. The blot module was held together firmly and slid into the guide rails on the lower buffer chamber. The module was locked into position. The blot module was filled with the 1X transfer buffer until the gel/membrane assembly was covered. The outer chamber was filled with 650 ml of deionised water. Transfer was performed using 30V for at least 1 hr, 170mA; or 15V overnight at 4° C.

2.2.7.3 Immunodetection

WesternBreeze® Chromogenic Western Blot Immunodetection kit (Invitrogen) was used to detect primary antibodies immobilised on PVDF membrane, following the manufacturer's instructions. For contents of kit (see Table 2.3 below).

Table 2.3: The contents of the immunodetection kit

Item	Description
Blocker/Diluent (part A)	Concentrated buffered saline solution containing detergent
Blocker/Diluent (part B)	Concentrated Hammersten casein solution
Antibody wash (16X)	Concentrated buffered saline solution containing detergent
Chromogenic substrate	Ready-to-use solution of BCIP/NBT substrate for alkaline phosphatase
Secondary antibody solution	Ready-to-use solution of alkaline phosphatase-conjugated, affinity purified, anti-rabbit IgG: goat conjugate

5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitro blue tetrazolium (NBT)

Membrane preparation

After protein transfer from the gels to the PVDF membranes, the membrane was washed 2X for 5 min with deionised water to remove gel and transfer buffer components and some weakly bound proteins. If the membrane was water-washed and dried, the membrane was re-wet in methanol followed by 2X water washes for 5 minutes before proceeding to immunodetection.

Solution preparation

Table 2.4: Table showing the components and the amounts used to prepare solutions for immunodetection assay

Solution	Components	Amounts (ml)
Blocking solution	Deionised water	5
	Blocker/diluent (part A)	2
	Blocker/diluent (part B)	3
	Total	10
Primary antibody diluent	Deionised water	7
	Blocker/diluent (part A)	2
	Blocker/diluent (part B)	1
	Total	10
Antibody wash	Deionised water	150
	Antibody washing solution (16X)	10
	Total	160

Immunodetection procedure

The membrane was placed in 10 ml blocking solution (Table 2.4) in a covered dish and incubated for 30 min on a shaker, followed by rinsing 2 X with deionised water for 5 min. The membrane was incubated in 10 ml of primary antibody solution (containing 1:50 – 1:200 antibody) for 1 hr followed by washing 4X (5 min each) with 20 ml of prepared antibody wash (Table 2.4). The membrane was then incubated in 10 ml of secondary antibody for 30 min followed by washing 4X (5 min each) with 20 ml of prepared antibody wash and 3X with deionised water. The membrane was then incubated in 5 ml of the chromogenic substrate until purple bands developed on the membrane – at least 1hr. This was followed by washing of the membrane 2X with deionised water. The membrane was then dried on a clean piece of filter paper to open air. After drying, the membrane was imaged using UVP BioImaging system using Labworks or scanned using EPSON scanner.

2.2.8 Zymography

Principle

The protease sample is denatured in SDS buffer and electrophoresed on a zymogram substrate containing gel using Tris-Glycine SDS running buffer. Following the run, the protease is renatured by incubating the gel in zymogram renaturing buffer containing a non-ionic detergent (Triton X-100). The gels are equilibrated and incubated in zymogram developing buffer in order to add the divalent metal cation required for enzymatic activity. During this incubation, the protease degrades the substrate impregnated in the gel, in this case gelatin. This is followed by staining and destaining of the gel such that protease bands appear as clear bands against a dark background. The dye used penetrates the entire gel, but only binds permanently to the proteins. Excess dye is washed by destaining, with first destaining solution acting by shrinking the gel thus squeezing out the liquid from the gel, and the final destaining solution swells the gel and enhances clearing of the gel.

Reagents and materials

Zymogram gelatin pre-cast gels (10 % Tris-Glycine gel with 0.1 % gelatin incorporated as a substrate); protein molecular weight marker; Tris-Glycine SDS sample buffer; 10X Tri-Glycine SDS running buffer (diluted 10X in deionised water prior to use); 10X Zymogram renaturing buffer (diluted 10X in deionised water prior to use); and 10X Zymogram developing buffer (diluted 10X in deionised water prior to use) (all purchased from Invitrogen) were used in the zymography studies.

Gel preparation

The gel cassettes were removed from the pockets and washed with deionised water, the slot on the gel was stripped open and the comb was removed. The wells were washed with the Tris-Glycine running buffer 3X and filled with the buffer.

Sample preparation and electrophoresis

Conditioned media from chondrocytes or explant cultures were used. 5 µl of Novex® Tris-Glycine SDS sample buffer was added to the samples. No reducing agent was added to the samples so that proteases would maintain their subunit assemblies that they might need for activity. The samples were not boiled prior to electrophoresis.

When loading samples into the wells, the marker was separated from the sample lanes by one or two lanes to prevent diffusion of reducing agent which may interfere with the protease activity. The same marker as in the immunoblotting experiments was used and so were the loading volumes. The gel cassettes were loaded onto the electrophoresis module of the XCell *SureLock*® Mini-Cell. The 1X Tri-Glycine SDS running buffer was used to fill both the upper (200 ml) and lower chamber (600 ml). The gels were run at 125 V and 40 mA for 90 minutes. The gels were removed from the cassettes after electrophoresis by opening the cassettes using a gel knife. The gel

rested on one of the plates of the cassette and the gel was wetted with the renaturing buffer to avoid drying. A piece of clean parafilm was placed on the gel and it was turned over so the gel rested on the piece of parafilm. The gel was then transferred to a dish for development.

Developing zymogram gels

After electrophoresis, the gel was incubated in 1X zymogram renaturing buffer for 30 min at room temperature on a shaker. The renaturing buffer was decanted and the gel was equilibrated in 1X developing buffer for 30 min at room temperature on a shaker. The buffer was decanted and fresh 1X developing buffer was added and the gel was incubated overnight at 37 °C.

Staining zymogram gels

Staining solution

0.1g Coomassie Brilliant Blue – R-250 (0.1%); 50 ml methanol (50 %) and 10 ml glacial acetic acid (10%) were combined and made up to 100 ml with deionised water. The solution was filtered prior to use.

Destaining solution

50 ml methanol (50 %) and 10 ml glacial acetic acid (10%) were combined and made up to 100 ml with deionised water.

Final destaining solution

7 ml methanol (7 %) and 10 ml glacial acetic acid (10%) were mixed and made up to 100 with deionised water.

Procedure

After development, the gels were washed in a solution containing 50 % methanol and 10 % acetic acid for 30 min at room temperature. The gels were placed in the staining solution about 3 hrs or overnight on a shaker. The

gels were removed from the staining solution and placed in destaining solution for a few hours (changing the solution as necessary) until the protease activity bands were clear. Destaining was finished with the solution containing 7 % methanol and 10 % acetic acid. Finally the gels were washed in deionised water. The gel was then placed between two pieces of parafilm and was imaged using the UVP BioImaging system using Labworks software or scanned using EPSON scanner.

2.2.9 Immunofluorescence

Basic principle

Immunofluorescence, which is immunocytochemistry with fluorescence detection, is a technique whereby an antibody labelled with a fluorescent molecule is used to detect the presence of an antigen in or on a cell or tissue by the fluorescence emitted by the bound antibody. This could be done directly or indirectly. In this study, the indirect immunofluorescence was used, in which the antibody specific for the antigen was unlabeled and a second anti-immunoglobulin antibody directed toward the first antibody was conjugated to the fluorochrome, fluorescein isothiocyanate (FITC). Fluorescence was detected using a fluorescence microscope. The sample was excited with a blue light; and following the excitement, the cell target protein labelled with FITC emitted a bright green (fluorescent) colour. This analysis was carried out as described by Smith *et al.* (1985)

Cell fixation

At the end of culture period, cells grown on coverslips or chamber slides were washed with PBS. The cells were fixed in 4 % paraformaldehyde for 20 min to preserve them and their cellular architecture. The cells were washed and stored in PBS at 4 °C until further analysis. To prepare 4 % paraformaldehyde, PBS (without Ca²⁺ and Mg²⁺) was added to 8 g of paraformaldehyde and made up to 100 ml. The mixture was heated in a water bath at 70 °C until the solution became clear. The solution was filtered,

allowed to cool at room temperature and kept at 4 °C. It was diluted 2X in PBS prior to use.

Immunostaining

The cells were washed in PBS and permeabilised with 0.1 % Triton X-100 (in PBS) for 15 min at room temperature, followed by washing in PBS. Non-specific binding sites were blocked with 2 % normal goat serum (NGS) in PBS for 1 hr at 37 °C. The cells were covered with primary antibody diluted in 2 % NGS (in PBS) overnight at 4 °C. Following the overnight incubation the cells were washed in PBS (containing 0.05 % Tween 20) once quickly and for 20 min on a shaker. The fluorescently labelled secondary antibody diluted 1:100 in 2 % NGS (in PBS) was added to the cells and they were incubated covered with aluminium foil for 1 hr at room temperature. The cells were washed in PBS (containing 0.05 % Tween 20) once quickly and for 20 min on a shaker and they were equilibrated with a buffer provided with the slow anti-fade mounting kit and mounted in slow anti-fade mounting medium (Molecular Probes). The coverslips were sealed with clear nail polish. The cells were viewed and imaged using a fluorescence microscope coupled to a Coolsnap digital camera.

2.2.10 The Fast Activated Cell-based ELISA (FACE®) for p38 Mitogen-Activated Protein Kinase (MAPK)

Principle of the assay

In FACE®, cells were cultured in 96-well plates and stimulated to induce the p38 MAPK pathway. Following 15-30 min stimulation, the cells were fixed rapidly to preserve activation-specific protein modifications. Each well was then incubated with a primary antibody specific for the activated p38 MAPK. Subsequent incubation with secondary HRP-conjugated antibody and developing solution produced a coloured (blue) product. The reaction was stopped using sulphuric acid which turned the blue solution to yellow one, the absorbance of which at 450 nm was related to the amount of p38 MAPK. The

number of cells in each well was normalized with the crystal violet solution provided. FACE® p38 Kit also contained a primary antibody specific for the native inactive p38 MAPK, so both native and activated protein levels were monitored in the same experiment.

Components of the FACE® p38 MAPK kit (see Table 2.5)

Table 2.5: Components of the FACE p38 MAPK kit

Reagents	Quantity (per plate)	Preparation
Phospho-p38 antibody	9 µl	1/500 dilution in antibody dilution buffer
Total-p38 antibody	9 µl	1/500 dilution in antibody dilution buffer
Anti-rabbit HRP-conjugated IgG	11 µl	1/2000 in antibody dilution buffer
1X antibody blocking buffer	22 ml	Ready to use
1X antibody dilution buffer	30 ml	Ready to use
10X PBS	120 ml	1/10 dilution with deionised water
10X Triton X-100	9 ml	1/100 dilution with PBS (0.1% Triton X-100)
Crystal violet solution	22 ml	Ready to use
Developing solution	22 ml	Ready to use
Stop solution	22 ml	Ready to use
1% SDS solution	22 ml	Ready to use
96-well tissue culture plate	2	Ready to use
Plate sealing tape	2	Ready to use
Additional Reagents		
10 % hydrogen peroxide (H ₂ O ₂)	3 ml	1/10 dilution with wash buffer (quenching buffer)
4 % paraformaldehyde	2.5 ml	

Procedure for the FACE® p38 assay

Cells were seeded in the 96-well plate at 2×10^5 cells/cm². The chondrocytes were grown for 5 days in complete medium. The medium was changed to one without FBS. The cells were stimulated for 15 or 30 min with IL-1 α (0.06nM) with or without Win-55,212-2 (1-10 μ M). The cells were fixed by replacing culture medium with 100 μ l of 4 % paraformaldehyde in PBS and the plate was incubated for 20 min at room temperature. This was followed by washing 3X with 200 μ l wash buffer. Each wash was done for 5 min on a shaker. 100 μ l quenching buffer (wash buffer with 1% H₂O₂) was added to the cells and incubated for 20 min at room temperature. The cells were washed 2X, 5 min each, with 200 μ l wash buffer. 100 μ l of antibody blocking buffer was added to the wells and incubated for 1 hr.

Half of the 96-well plate wells were incubated with diluted phospho-p38 antibody and the remaining half with total-p38 antibody. Some of the wells for each group were incubated with secondary antibody alone (negative controls). For negative control wells, cells were incubated with 40 μ l antibody dilution buffer during the primary antibody incubation step. Blocking buffer was removed and the cells were washed 2X with 200 μ l wash buffer. 40 μ l of diluted primary antibody (or antibody dilution buffer for negative control wells) were added to the cells and the plate was sealed with sealing tape and incubated overnight at 4° C. This was followed by washing 3X, 5 min each, with 200 μ l wash buffer. 100 μ l diluted secondary antibody was added and the plate was covered and incubated for 1 hr at room temperature. During this incubation, developing solution was transferred to a secondary container, protected from light by covering it with aluminium foil.

The cells were washed 3X, 5 min each, with 200 μ l wash buffer and then 2X, 5 min each, with 200 μ l 1X PBS. The PBS was removed and 100 μ l of developing solution were added to each well. The plate was incubated for 20 mins at room temperature, protected from light. Development of blue colour was monitored to avoid over-development. 100 μ l of stop solution was added.

This acidic solution turned the blue colour to yellow. Absorbance was read within 5 min at 450 nm.

Crystal Violet cell staining

After readings at 450 nm were complete, wells were washed 2X with 200 μ l wash buffer and 2X with 200 μ l 1X PBS. The plate was tapped on paper towels and air-dried at room temperature for 5 min. 100 μ l of crystal violet solution were added to each well and incubated for 30 min at room temperature. The wells were washed 3X with 200 μ l 1X PBS for 5 min each. 100 μ l of 1% SDS solution was added to each well and incubated on a shaker for 1 hr at room temperature. Absorbance was read at 570 nm. The measured absorbance at 450 nm was corrected for cell number by dividing the reading for a given well by the absorbance at 570 nm for that well.

2.2.11 Statistical analysis

All data was presented as mean \pm S.E.M. (standard error of the mean). Statistical differences from appropriate controls were determined using Kruskal-Wallis non-parametric test followed by Dunn's post-hoc test or one-way ANOVA followed by Boniferroni's post-hoc test and a $p < 0.05$ was considered statistically significant. The GraphPad Prism statistical package was used (GraphPad Software, Inc).

CHAPTER 3

CYTOTOXICITY ASSAYS IN CHONDROCYTE AND CARTILAGE EXPLANT CULTURES.

3.1 Introduction

The MTT tetrazolium salt colorimetric assay previously described by Mossmann (1983) to measure cytotoxicity, cell proliferation and cell activation was used to examine the effects of cannabinoids and other inhibitors on cellular viability. MTT yellow solution is converted to blue formazan in living cells. The formazan crystals were dissolved using acid-isopropanol. The absorbance measured was proportional to mitochondrial enzyme activity and viable cell numbers. CV solution was also used to verify the findings with the MTT assay. CV binds to cell nuclei and the absorbance of this stain at 550 nm is proportional to cell numbers.

When cells are damaged, they become 'leaky' and this phenomenon is determined by measuring LDH in the extracellular medium. This enzyme is normally present in the cytosol, and cannot be measured extracellularly unless cell damage has occurred. Therefore LDH release assays were performed to quantify the membrane-damaging effects of the cannabinoids on chondrocytes in cartilage explants.

3.2 Materials and methods

3.2.1 Materials: The endogenous cannabinoids, anandamide and N-arachidonyl glycine (N-AG); the synthetic cannabinoids ACEA, Win-55,212-2, HU-210 and JWH-133; and the antagonists, AM 281 and AM 630 which are also inverse agonists of cannabinoid receptors CB₁ and CB₂ respectively, were studied along with their solvent controls. Absolute ethanol (1.0 - 0.05% final concentration) and dimethyl sulfoxide (DMSO) (1.0 - 0.05% final concentration) were used as the solvents according to instructions from the suppliers of the drugs. Different inhibitors were also studied, including: NS-398, inhibitor of cyclooxygenase 2 (COX-2); ODQ, inhibitor of NO-sensitive guanylyl cyclase; and pyrrolidine dithiocarbamate ammonium (PDTC), inhibitor of NF- κ B.

3.2.2 Bovine chondrocyte culture: Bovine articular chondrocytes were cultured as described in section 2.2.1. When the chondrocytes reached near confluence (about 7-10 days) they were stimulated with IL-1 α (100 U/ml \equiv 0.06 nM) (Buttle *et al.*, 1997) and were incubated with the drugs for 48 h in DMEM with the supplements except FBS

3.2.3 Cartilage explant culture: Bovine nasal cartilage explants were cultured as described in section 2.2.2. The explants were stimulated with IL-1 α (500 U/ml \equiv 0.3 nM) (Buttle *et al.*, 1997) and were incubated with the drugs for 4 days in DMEM with the supplements except FBS

3.2.4 MTT assay: After 48 h incubation of the chondrocytes stimulated or unstimulated with IL-1 in the presence of the drugs, the MTT assay was performed as described in section 2.2.3.1.

3.2.5 Crystal violet assay: The CV assay was carried on chondrocytes following the 48 hr-incubation in the presence or absence of IL-1 \pm the drugs. For the rest of the assay procedure refer to section 2.2.3.2.

3.2.6 LDH release assay: conditioned media from bovine nasal cartilage explants incubated for 4 days in the presence or absence of IL-1 \pm anandamide or Win-55,212-2 at 100 μ M were used in the LDH release assay carried out as described in section 2.2.3.3.

3.2.7 Statistical Analysis: Kruskal-Wallis followed by Dunn's post-hoc test and ANOVA followed by Boniferroni's post-hoc test were used to determine statistical significance.

3.3 Results

3.3.1 Anandamide and Win-55,212-2 cytotoxicity studies on bovine articular chondrocytes

Chondrocytes were incubated in the presence or absence of IL-1 α (0.06 nM) \pm anandamide or Win-55,212 at 1-100 μ M. Their effects were compared with the appropriate vehicles, ethanol for anandamide and dimethyl sulfoxide (DMSO) for Win-55,212-2 at concentrations equivalent to those in the drug's solution. These vehicle treatments served as controls. The cells were incubated for 48 hrs followed by the analyses of cytotoxicity of the drugs using the crystal violet (CV) assay and MTT assay.

Anandamide significantly ($p < 0.001$) reduced cell viability at 100 μ M as shown in both the CV and the MTT assays (Fig. 3.1 and 3.2). Win-55,212-2 significantly affected cell viability negatively at 50 and 100 μ M ($p < 0.05$) as shown by the MTT and CV assays, respectively (Fig. 3.3 and 3.4).

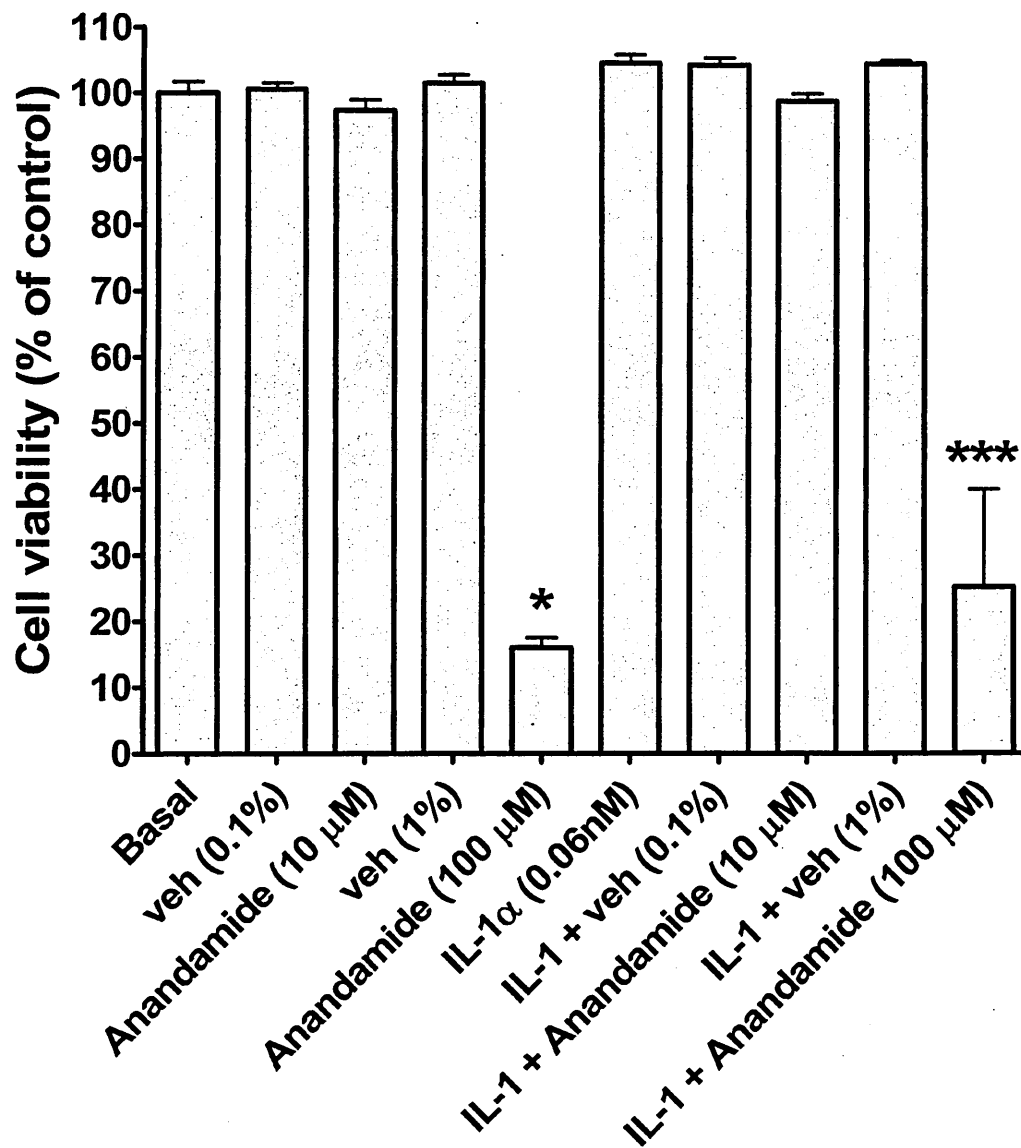


Figure 3.1: CV assay results showing the effect of 10-100 μM anandamide in primary bovine articular chondrocytes. Chondrocytes were incubated for 48 hrs in the presence or absence of IL-1α +/- anandamide or vehicle (ethanol). Absorbance corresponded to the number of viable chondrocytes and results are expressed as mean (% of basal) ± s.e.m. (n=6) * p<0.05; *** p<0.001 compared with vehicle control. Kruskal-Wallis followed by Dunn's test was used to determine significance.

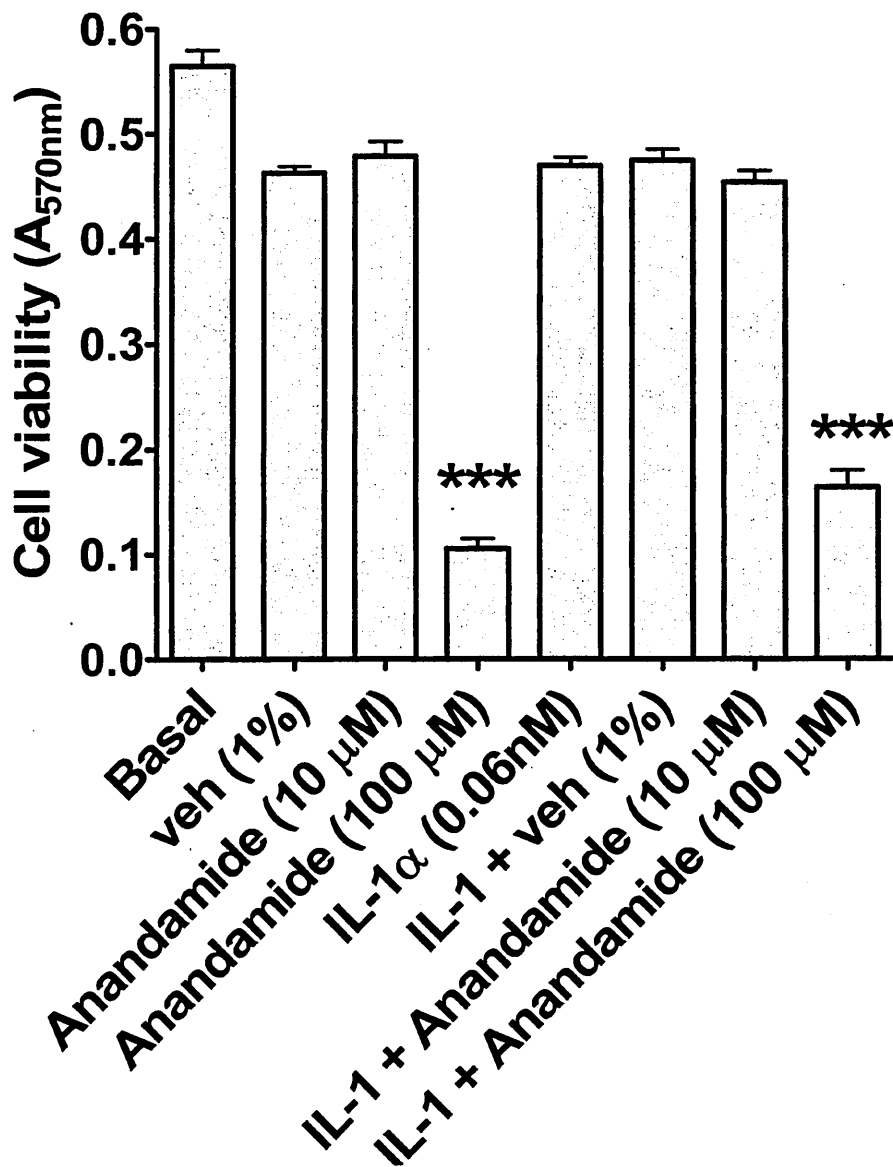


Figure 3.2: MTT assay results showing the effect of 10-100 μ M anandamide in primary bovine articular chondrocytes. Chondrocytes were incubated for 48 hrs in the presence or absence of IL-1 α +/- anandamide or vehicle (ethanol). Absorbance corresponded to the number of viable chondrocytes. Results are expressed as mean \pm s.e.m. (n=6) *** p<0.001 compared with vehicle control. ANOVA followed by Boniferroni post-hoc test was used to determine significance.

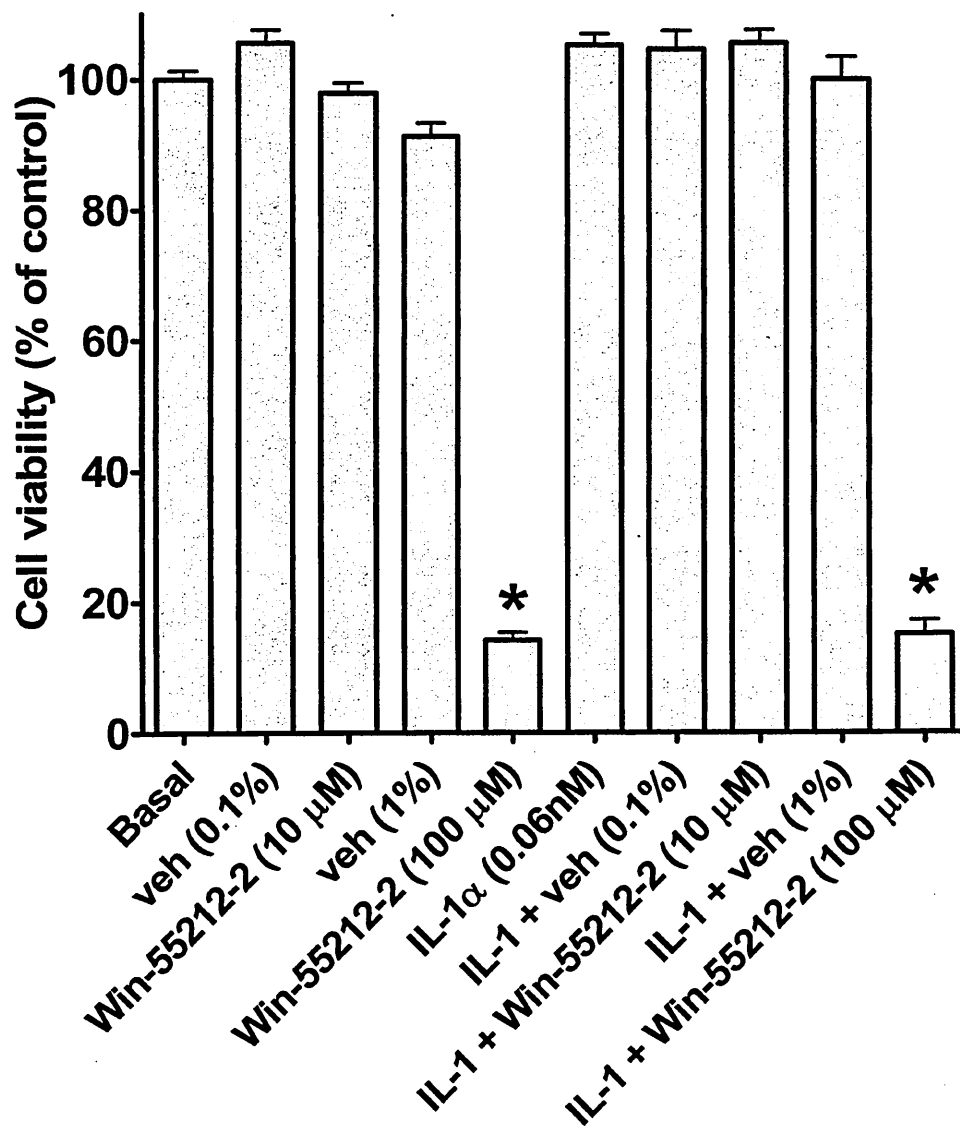


Figure 3.3: CV assay results showing the effect of 10-100 μ M Win-55,212-2 in primary bovine articular chondrocytes. Chondrocytes were incubated for 48 hrs in the presence or absence of IL-1 α +/- Win-55,212-2 or vehicle (DMSO). Absorbance corresponded to the number of viable chondrocytes and results are expressed as mean (% of basal) \pm s.e.m. (n=6) * p<0.05 compared with vehicle control. Kruskal-Wallis followed by Dunn's test was used to determine significance.

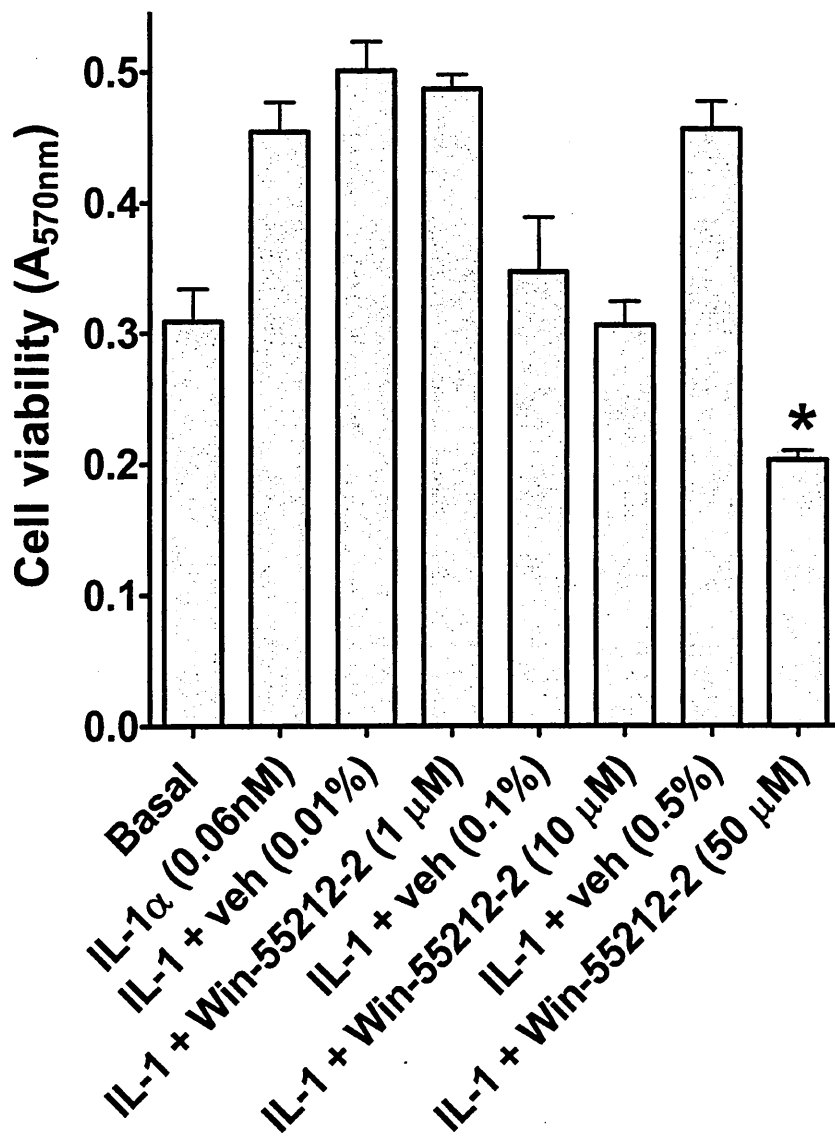


Figure 3.4: MTT assay results showing the effect of 1-50 μ M Win-55,212-2 in primary bovine articular chondrocytes. Chondrocytes were incubated for 48 hrs in the presence or absence of IL-1 α +/- Win-55,212-2 or vehicle (DMSO). Absorbance corresponded to the number of viable chondrocytes. Results are expressed as mean \pm s.e.m. (n=6) * p<0.05 compared with vehicle control. ANOVA followed by Boniferroni post test was used to determine significance.

3.3.2 Studies on the effects of anandamide and Win-55,212-2 on bovine nasal cartilage explants cytotoxicity (LDH release assay)

For this assay bovine nasal cartilage explants were cultured stimulated or unstimulated with IL-1 in the presence of anandamide and Win-55,212-2 at

100 μ M or their appropriate vehicles for 4 days. Cytotoxicity of the cannabinoids was determined by the LDH release assay.

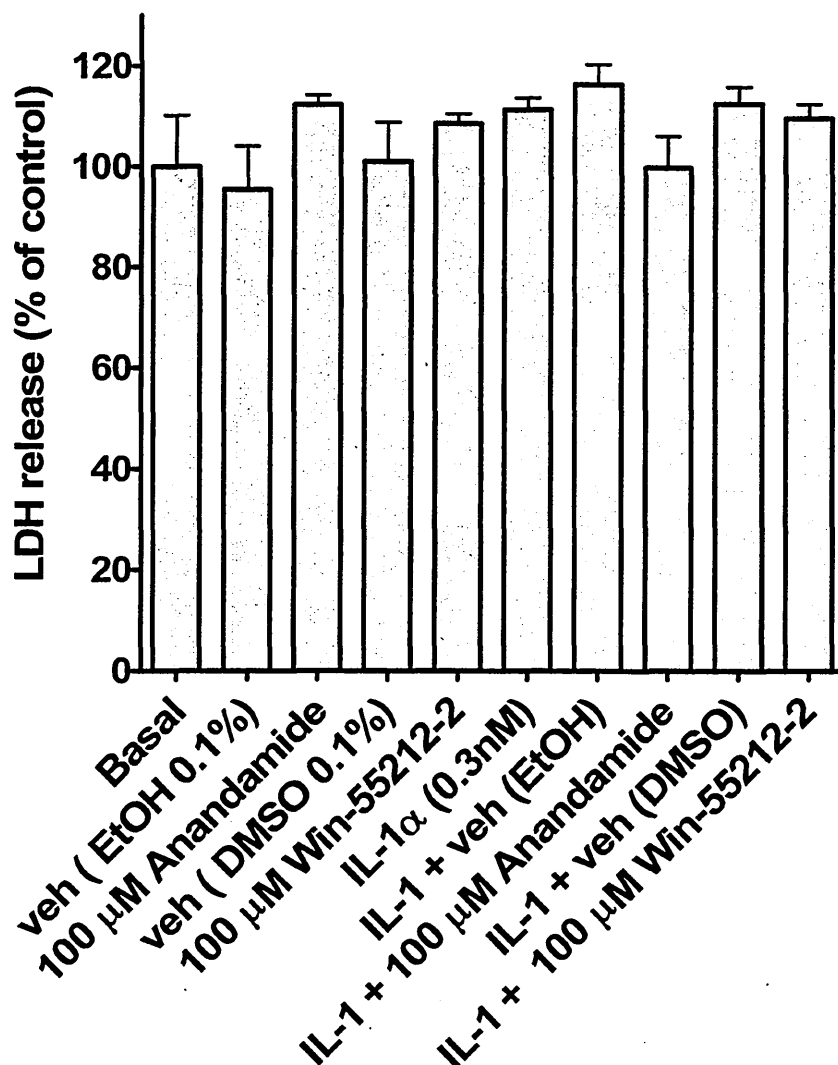


Figure 3.5: The results of the LDH release studies on 100 μ M anandamide and 100 μ M Win-55,212-2 in bovine cartilage explants. The explants were incubated for 4 days in the presence or absence of anandamide/Win-55,212-2 or the vehicles (ethanol/DMSO) +/- IL-1 α . The results are expressed as mean (% of basal) \pm s.e.m. (n=6)

IL-1 and the vehicles did not significantly reduce cell viability in the cartilage explant cultures compared with the basal. The cannabinoids, anandamide and Win-55212-2 also did not significantly affect viability of chondrocytes in

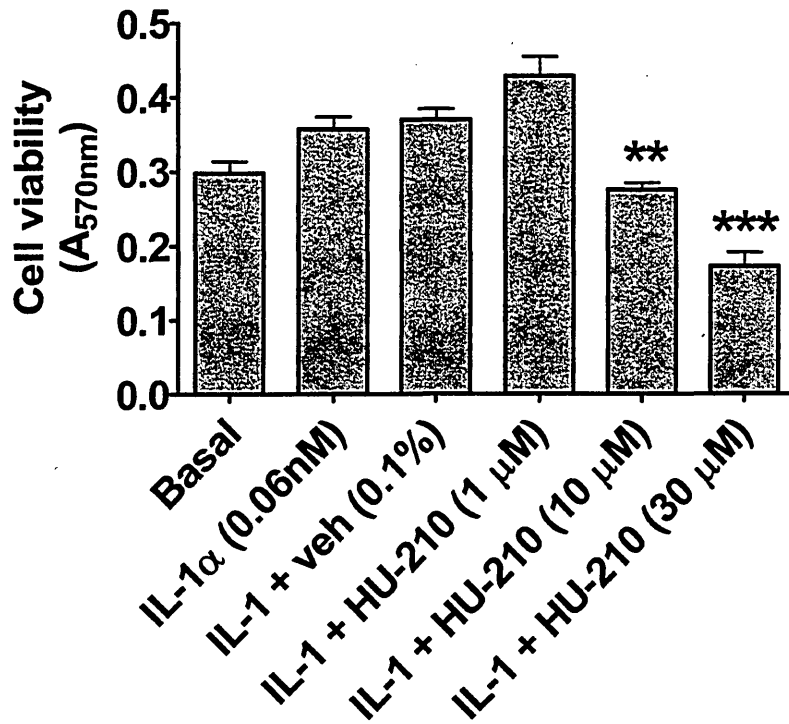
this system at 100 μM compared with both the basal and vehicle controls (Fig. 3.5).

3.3.3 Studies on synthetic cannabinoid HU-210 and antagonists AM 281 and AM 630 on chondrocytes cytotoxicity.

Effects of synthetic cannabinoid, HU-210 and the cannabinoid receptor CB_1 and CB_2 antagonists, AM 281 and AM 630, respectively on chondrocytes viability were studied. Bovine articular chondrocytes were incubated for 48 hrs in the presence or absence of IL-1 \pm HU-210 (1-30 μM), AM 281 (10-100 μM), AM 630 (10-100 μM) or their vehicle (ethanol, 0.1% final concentration). Cell viability was determined by using the MTT assay.

IL-1 and vehicle (ethanol) for experiments did not affect chondrocytes viability. However, HU-210 appeared to significantly reduce cell viability from 10 μM (Fig. 3.6a). The antagonists AM 281 and AM 630 did not affect cell viability even at the high concentration of 100 μM (Fig. 3.6b).

(a)



(b)

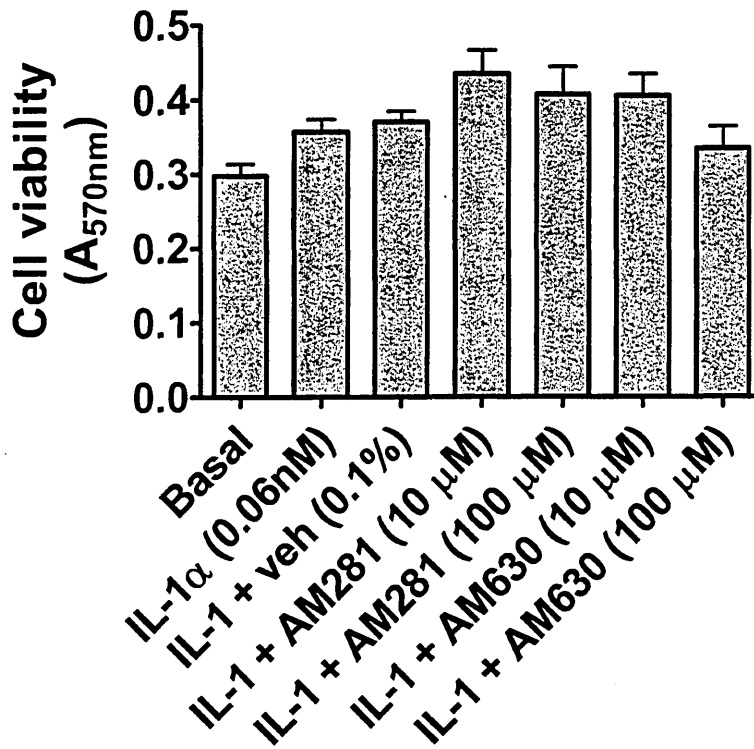


Figure 3.6 a, b: MTT Assay results showing the effect of (a) HU-210 (1-30 μ M) and (b) antagonists, AM281 (10-100 μ M) and AM630 (10-100 μ M) on IL-1 α -stimulated bovine primary articular chondrocytes. Results expressed as mean \pm s.e.m. (n=6), ** p<0.01; *** p<0.001.

3.3.4 Cytotoxicity studies of cannabinoids ACEA, N-AG and JWH-133 on bovine articular chondrocytes

Endocannabinoid N-arachidonylglycine (N-AG), synthetic cannabinoids ACEA (CB₁ receptor specific agonist) and JWH-133 (CB₂ receptor specific agonist) were studied to determine whether they may affect chondrocyte viability. IL-1-stimulated chondrocytes were incubated with these cannabinoids at 10-50 μ M or their vehicle (ethanol) at 0.1% final concentration, for 48 hrs and MTT assay was carried out on the chondrocytes.

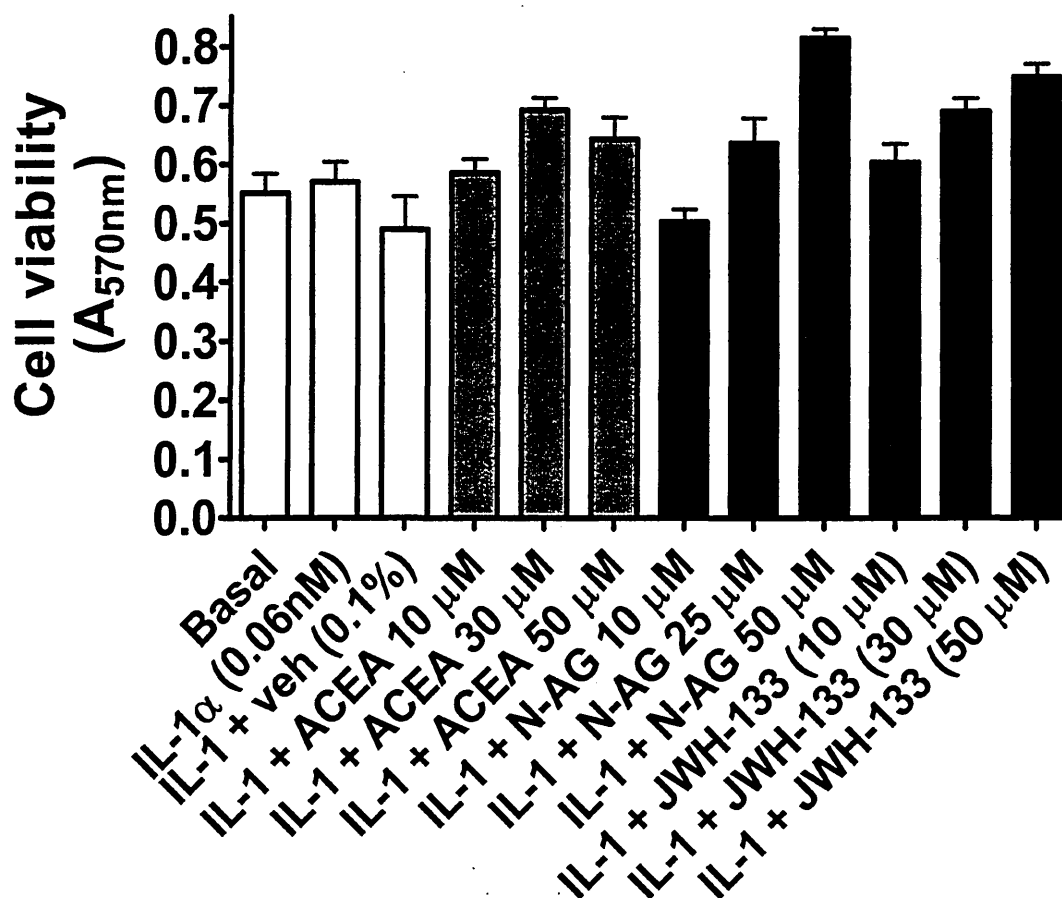


Figure 3.7: MTT Assay results showing the effect of ACEA (10, 30 and 50 μ M); N-AG (10, 25 and 50 μ M); and JWH-133 (10, 30 and 50 μ M) on IL-1 α -stimulated bovine primary articular chondrocytes incubated for 48 hrs. The results are expressed as mean \pm s.e.m. (n=6).

The cannabinoids, ACEA, N-AG and JWH-133 at the concentrations up to 50 μM did not significantly affect cell viability compared with the controls (Fig. 3.7).

3.3.5 Cytotoxicity studies of the inhibitors ODQ and PDTC on bovine articular chondrocytes

Cytotoxic effects of ODQ, the inhibitor of NO-sensitive GC and PDTC, the inhibitor of NF- κB were studied on chondrocytes stimulated with IL-1 for 48 hrs.

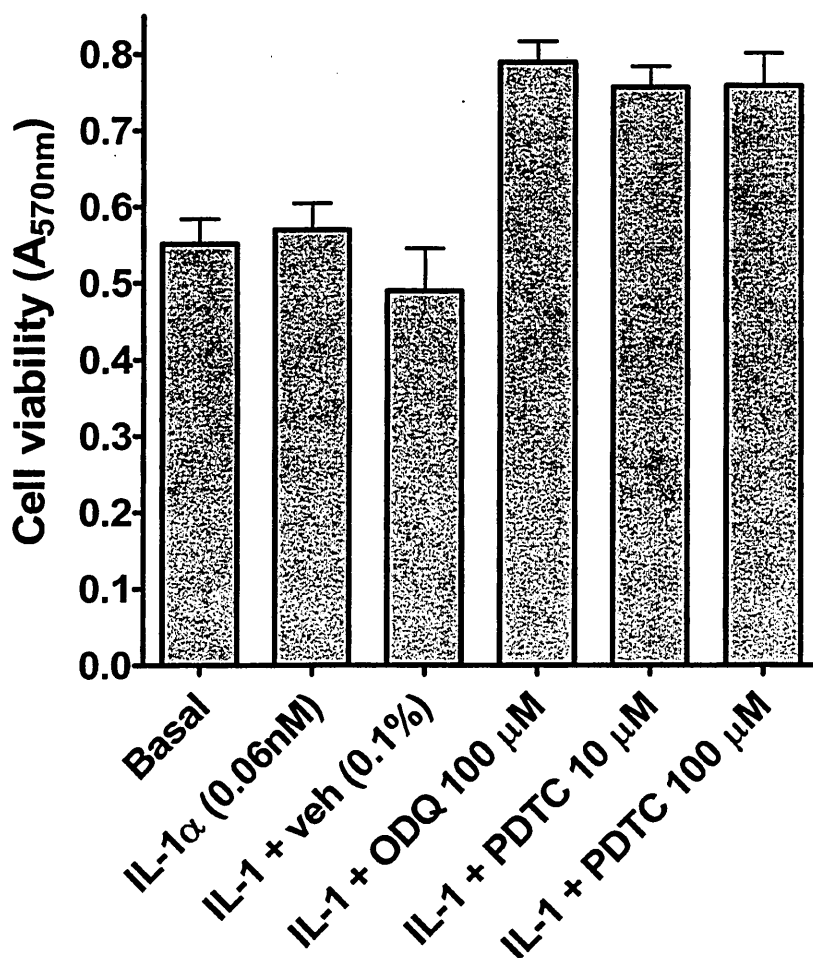


Figure 3.8: MTT Assay results showing the effect of ODQ (100 μM); PDTC (10 and 100 μM) on IL-1 α -stimulated bovine primary articular chondrocytes incubated for 48 hrs. The results are expressed as mean \pm s.e.m. (n=6).

ODQ and PDTC did not affect cell viability negatively but appeared to enhance proliferation when compared with basal, IL-1 and vehicle controls (Fig. 3.8).

3.4 Discussion

The cytotoxicity studies showed that most of the cannabinoids and inhibitors used are not toxic to the chondrocytes, except for anandamide, HU 210 and Win-55,212-2 which may be toxic to bovine chondrocytes at concentrations equal to or greater than 10 μM (Fig. 3.2 and Fig. 3.6a). Cannabinoids appear to be toxic at 100 μM in chondrocytes, although at the same concentrations showed no significant reduction of cell viability in cartilage explant cultures (Fig. 3.5). It appears that chondrocytes embedded in the ECM may be protected from the cytotoxic effects of the cannabinoids. The antagonists used did not induce cell death even at a concentration of up to 100 μM (Fig. 3.6b). ODQ and PDTC also did not reduce cell viability.

High concentrations of cannabinoids were used in the cytotoxicity assays to establish the upper limits of the range of concentrations used. It was important to ensure that the results observed in other investigations such as reduction of inflammatory mediators' expression and production were not due to reduced cell viability.

CHAPTER 4

EFFECTS OF CANNABINOIDS ON IL-1-INDUCED NO PRODUCTION IN ARTICULAR CHONDROCYTES

4.1 Introduction

Incubation of human articular chondrocytes with IL-1 results in the time-dependent expression of the enzyme induced NO synthase (iNOS), which appeared to be conserved between different cell types and across species (Charles *et al.*, 1993) and this iNOS (\approx 131 kDa) was one of the first to be cloned and sequenced. Research on the biological roles of NO has revealed that it functions as an important signal and effector molecule in a variety of physiologic and pathologic settings (Stuehr, 1997). Superficial bovine cartilage explants and cells stimulated with IL-1, LPS, or TNF- α for 24 and 48 hours were shown to produce, significantly, more NO than did deep explants and cells treated the same (Hayashi *et al.*, 1997). This apparent increase of NO production at the cartilage surface-synovial fluid interface may play an important role in the modulation of cartilage damage in inflammatory arthritis (Hayashi *et al.*, 1997). Articular chondrocytes are the major source of inducible nitric oxide synthase and nitric oxide during inflammation or infection of a joint (Murrell *et al.*, 1996). In osteoarthritic tissue, iNOS is the enzyme that is responsible for the increased production of NO (Alderton *et al.*, 2001). In articular chondrocytes and cartilage, NO plays a regulatory role in the activation of metalloproteinases (Murrell *et al.*, 1995) and thus may be involved in cartilage catabolism. Inhibition of NO production in the cartilage tissue may be a potential target in the treatment of arthritis.

Cannabinoids have been shown to be capable of inhibiting iNOS transcription and NO production in macrophage cell line (RAW 264.7), rat microglial cells and neuronal cells at micromolar range concentrations (Hillard *et al.*, 1999; Jeon *et al.*, 1996 ; Waksman *et al.*, 1999). Therefore the present study aimed at testing the hypothesis that cannabinoids may inhibit IL-1-induced NO production in articular chondrocytes by inhibiting iNOS expression in these cells.

4.2 Materials and Methods

4.2.1 Cannabinoids and drugs: The endocannabinoids anandamide, N-Arachidonyl glycine (N-AG) and NADA; the synthetic cannabinoids ACEA, Win-55,212-2, Win-55,212-3, HU-210 and JWH-133; and the antagonists, AM281 and AM630 which are also inverse agonists of cannabinoid receptors CB₁ and CB₂ respectively, were studied along with their vehicle controls. Absolute ethanol (0.1% final concentration) and dimethyl sulfoxide (DMSO) (0.1% final concentration) were used as the solvent according to instructions from the suppliers of the cannabinoids. The same was true for the different inhibitors studied, namely: MK-886, inhibitor of 5-lipoxygenase activating protein (FLAP); NS 398, inhibitor of cyclooxygenase 2 (COX-2); ODQ, inhibitor of NO-sensitive guanylyl cyclase; and pyrrolidine dithiocarbamate ammonium (PDTC), inhibitor of NF- κ B. Their effects were investigated on unstimulated or IL-1 α -stimulated chondrocyte NO production and iNOS expression at concentrations which were demonstrated to be non-toxic to chondrocytes.

4.2.2 Bovine chondrocyte culture: Bovine articular chondrocytes were cultured as described in section 2.2.1. After at least 5 days, chondrocytes were incubated with the drugs \pm IL-1 α (100U/ml \equiv 0.06nM) for 48h in DMEM with the supplements except FBS.

4.2.3 NO production determination: NO was measured as nitrite in primary bovine articular chondrocyte conditioned media as described under section 2.2.4.

4.2.4 Immunoblotting: The immunoblot analysis was carried out as describe in section 2.2.7. The whole cell lysates were applied to SDS-polyacrylamide pre-cast 4-12 % NuPAGE® Bis-Tris mini-gels (Invitrogen). SeeBlue plus2 pre-stained protein standard (Invitrogen) was used for easy and clear band identification. Then the proteins were electroblotted onto invitrolon® polyvinylidene difluoride (PVDF) membranes. WesternBreeze®

Chromogenic Western Blot Immunodetection kit (Invitrogen) was used to detect primary antibodies immobilised on PVDF membrane, following the manufacturer's instructions. Briefly, after blocking the membranes, they were incubated in primary antibody, rabbit polyclonal anti-inducible nitric oxide (iNOS) (1:500) (Alexis, UK) for 1 hr. After washing the membrane with the antibody wash, it was incubated in alkaline phosphatase-conjugated goat anti-rabbit secondary antibody for 30 min. After further washing with antibody wash and water, purple protein bands were visualized with chromogenic substrate [5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitro blue tetrazolium (NBT)] for 1hr.

4.2.5 Statistical Analysis: Kruskal-Wallis non-parametric test followed by Dunn's post-hoc test or one-way ANOVA followed by Boniferroni's post-hoc test were used to determine statistical significance. P-value of less than 0.05 was considered statistically significant.

4.3 Results

4.3.1 Effect of Win-55,212 alone or in combination with CB₁ antagonist AM281

Bovine primary chondrocytes were stimulated with IL-1 α (0.06 nM) to produce NO. Effects of a non-selective cannabinoid receptor agonist, Win-55,212-2 and effects of the antagonist which also acts as an inverse agonist at CB₁ cannabinoid receptor, AM 281, on IL-1-induced NO production were studied. AM 281 was expected to inhibit effects of Win-55,212-2, in treatments where these drugs were added together, to show that effects of Win-55,212-2 were mediated through the CB₁ cannabinoid receptor. AM 281 was also expected to exhibit effects that were opposite to those produced by Win-55,212-2 when applied to the cells without Win-55,212-2.

Win-55,212-2 at concentrations 0.5-10 μ M was used in this study. Win-55,212-2 at 1-10 μ M reduced the basal level of NO production whilst at 0.5 μ M increased this (Fig. 4.1a). Treatment of bovine articular chondrocytes with

IL-1 α (0.06 nM) significantly increased NO production in these experiments. Win-55,212-2 at 0.5 μ M did not have any significant effect but at 1-10 μ M significantly ($p < 0.001$) reduced NO production compared to the vehicle control, and 5-10 μ M reduced NO production to below basal levels. Chondrocytes unstimulated or stimulated with IL-1 α were also treated with 100 μ M AM 281 alone or in the presence of Win-55,212-2. AM 281 did not have any effect on unstimulated chondrocytes (Fig. 4.1a) but significantly reduced NO production in IL-1-stimulated chondrocytes compared with the vehicle controls ($p < 0.001$) (Fig. 4.1b). Treatment of IL-1-stimulated chondrocytes with a combination of Win-55,212-2 and AM 281, reduced their NO production further, below the effect of each component alone, and to the extent that even 0.5 μ M Win-55,212-2 in combination with AM 281 reduced NO production to basal level (Fig. 4.1b).

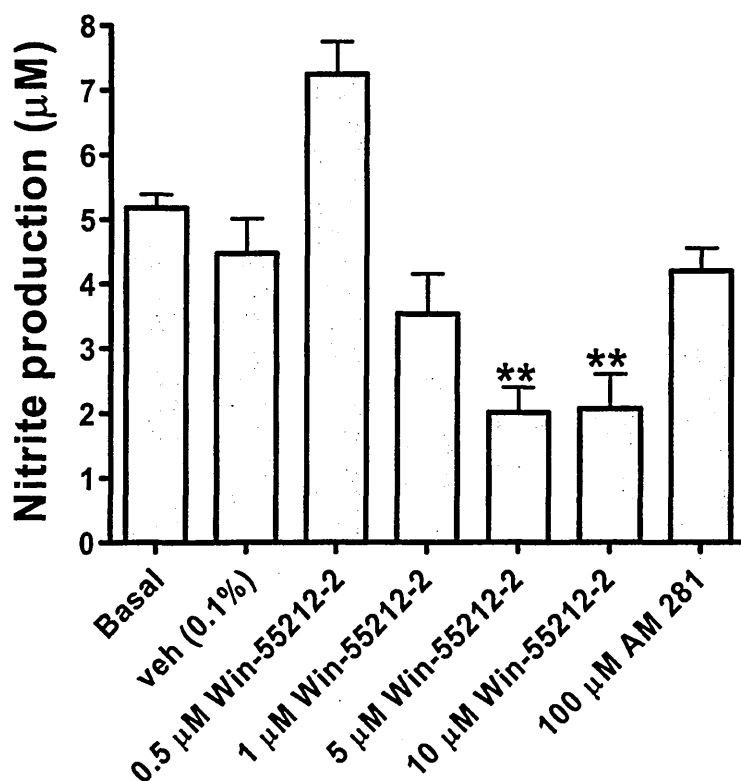


Figure 4.1a: Effects of Win-55,212-2 (0.5-10 μ M) and CB₁ cannabinoid receptor antagonist/inverse agonist, AM 281 (100 μ M), on basal NO production in bovine articular chondrocytes. The results are presented as mean \pm s.e.m. ($n=6$). ** $p < 0.01$ compared with appropriate vehicle controls.

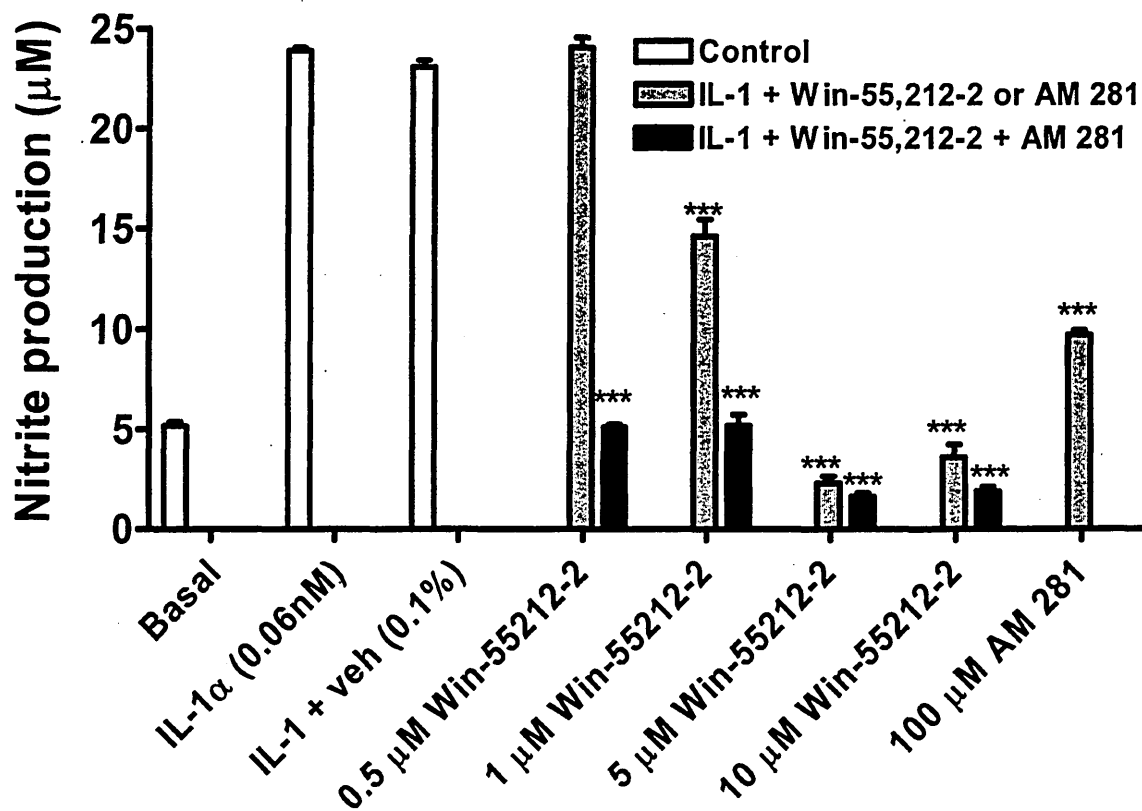


Figure 4.1b: Effects of Win-55,212-2 (0.5-10 µM) with or without CB₁ cannabinoid receptor antagonist/inverse agonist, AM 281 (100 µM), on IL-1α-induced NO production in bovine articular chondrocytes. The results are presented as mean ± s.e.m. (n=6). *** p<0.001 compared with appropriate vehicle controls.

4.3.2. Effect of Win-55,212-2 alone or in combination with CB₂ antagonist AM 630

Chondrocytes were unstimulated or stimulated with IL-1α (0.06 nM) to produce NO. Effects of a non-selective cannabinoid receptor agonist, Win-55,212-2 and effects of the antagonist which also acts as an inverse agonist at CB₂ cannabinoid receptor, AM 630, on IL-1-induced NO production were

studied. AM 630 was expected to inhibit effects of Win-55,212-2, in treatments where these drugs were added together, to show that effects of Win-55,212-2 were mediated through the CB₁ cannabinoid receptor. AM 630 was also expected to exhibit effects that were opposite to those produced by Win-55,212-2 when applied to the cells without Win-55,212-2.

Win-55,212-2 (1 and 10 μ M) and 100 μ M AM 630 with or without IL-1 α (0.06 nM) were used in these experiments (Fig. 4.2a, b). In unstimulated chondrocytes, Win-55,212-2, at 1 μ M had no effect on NO production but at 10 μ M significantly reduced basal NO production (Fig. 4.2a). AM 630 had no effect on basal NO production (Fig. 4.2a). When the chondrocytes were stimulated with IL-1 α , they produced significant amount NO. Win-55,212-2 at both concentrations significantly reduced this IL-1-induced NO production, with 10 μ M Win-55,212-2 reducing NO production below basal level (Fig 4.2b). AM 630 alone also reduced IL-1-induced NO production significantly compared to the vehicle control (Fig. 4.2b). 1 μ M Win-55,212-2 combined with AM 630, reduced IL-1-induced NO production further, but not as markedly as that with AM 281, while 10 μ M Win-55,212-2 in combination with AM 630 also reduced NO production significantly ($p < 0.001$) compared with the vehicle control (Fig. 4.2b).

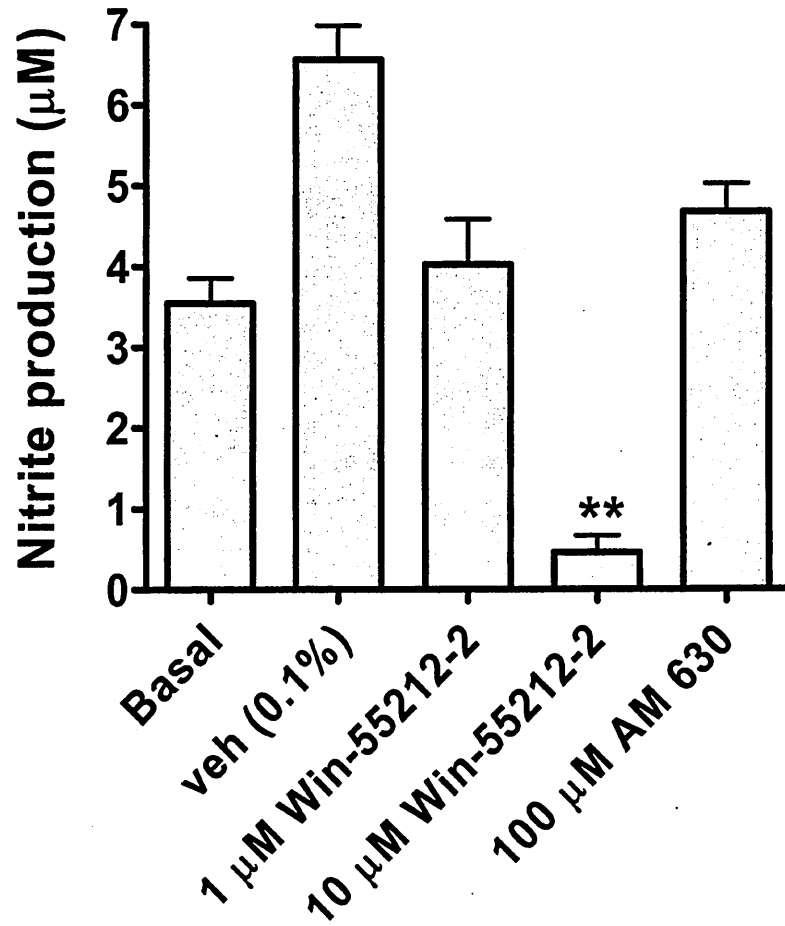


Figure 4.2a: Effects of Win-55,212-2 (1-10 µM) and CB₂ cannabinoid receptor antagonist/inverse agonist, AM 630 on basal NO production in bovine articular chondrocytes. Results presented as mean ± s.e.m. (n=6) ** p<0.01 compared with vehicle control.

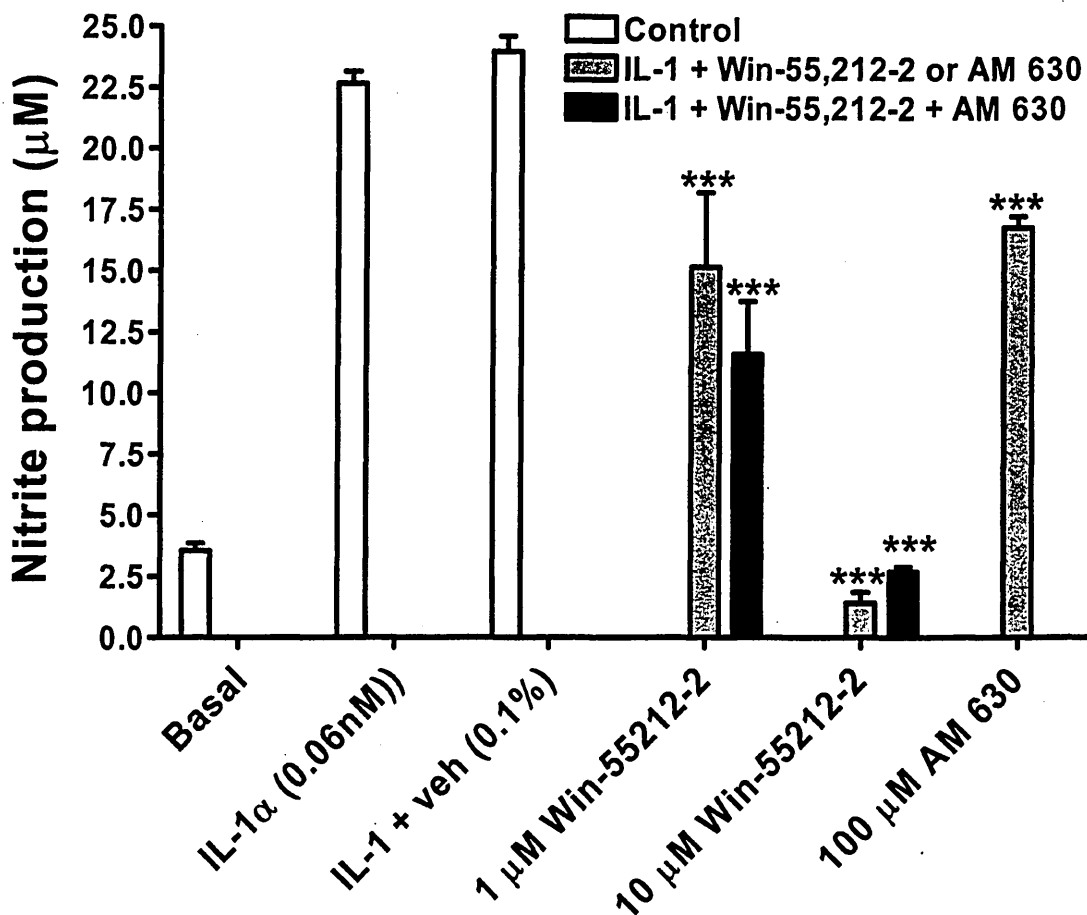


Figure 4.2b: Effects of Win-55,212-2 (1-10 μM) with or without CB_2 cannabinoid receptor antagonist/inverse agonist, AM 630 on IL-1 α -induced NO production in bovine articular chondrocytes. Results presented as mean \pm s.e.m. (n=6) ***p<0.001 compared with vehicle control.

4.3.3. Effect of Win-55,212-3 compared with Win-55,212-2

The two enantiomers, Win-55,212-3 (inactive) and Win-55,212-2 (active) were used to carry out comparative studies of their effects on NO production in bovine articular chondrocytes stimulated by IL-1. These studies would help shed some light on whether cannabinoids such as Win-55,212 produced their effects in chondrocyte by acting via receptor(s).

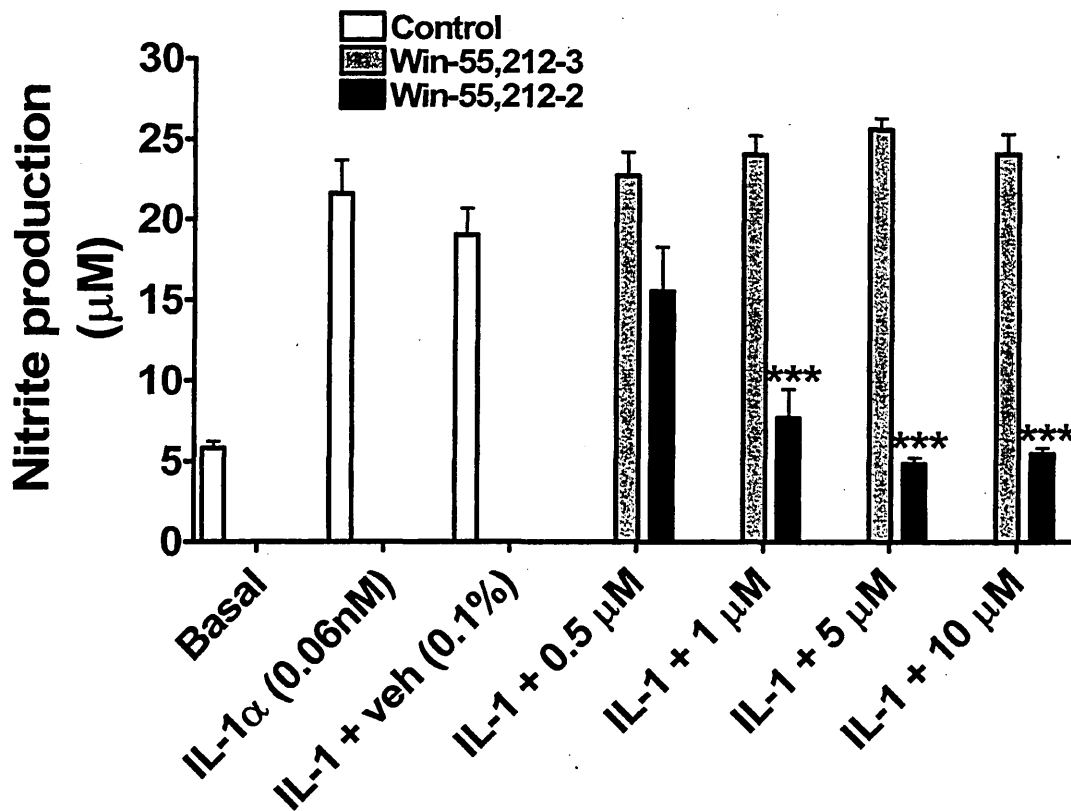


Figure 4.3: The Effect of Win-55,212-2 (active enantiomer) and Win-55,212-3 (inactive enantiomer) on IL-1 α -induced NO production in bovine articular chondrocytes. The results are presented as mean \pm s.e.m. (n= 6). ***p<0.001 compared with vehicle controls.

The inactive enantiomer, Win-55,212-3 did not have any significant effect on IL-1 α -induced NO production (Fig. 4.3). The active enantiomer, Win-55,212-2 at 1, 5 and 10 μ M, however, reduced IL-1 α -induced NO production to or near basal levels. These effect were statistically significant compared with the vehicle control (p<0.001).

4.3.4 Effect of HU-210 on IL-1-induced NO production

The synthetic cannabinoid HU-210, one of the potent cannabinoids was studied to find out whether it has the capacity to modulate IL-1-induced NO production in chondrocytes. The chondrocytes were incubated with HU-210

(1-10 μM) or its vehicle, ethanol (0.1% final concentration) for 48 hrs prior to nitrite assay.

HU-210 had no effect at 1 μM on IL-1-induced NO production but at 5 and 10 μM appeared to significantly reduce the NO production in bovine articular chondrocytes (Fig. 4.4).

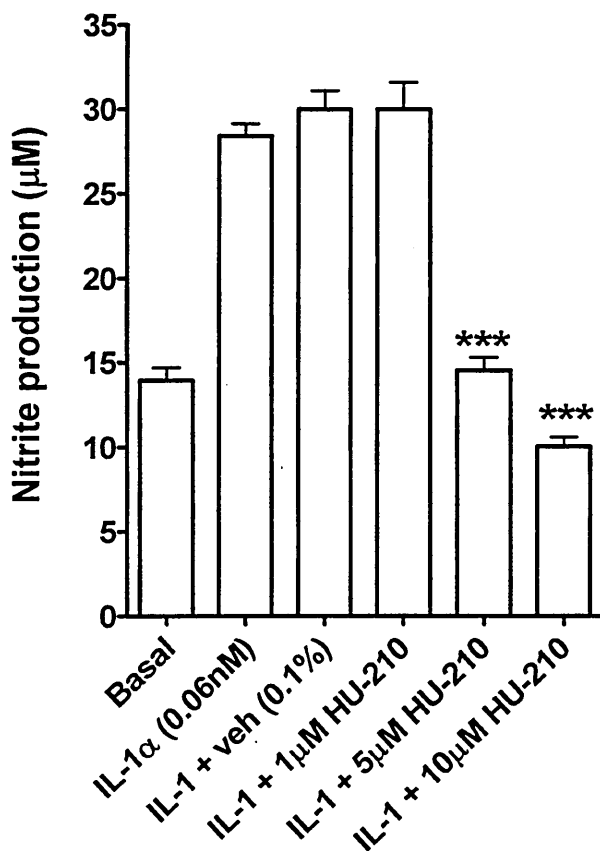


Figure 4.4: The Effect of HU-210 on IL-1 α -induced NO production in bovine articular chondrocytes. Results presented as mean \pm s.e.m. (n=6). *** $p < 0.001$ compared with vehicle controls. ANOVA followed by Boniferroni's post-hoc test was used to determine statistical significance.

4.3.5 Effects of other cannabinoids: anandamide and/or NADA; ACEA; N-AG; and JWH-133 on IL-1-induced NO production

Endocannabinoids, anandamide and NADA were studied alone or in combination on IL-1-induced NO production in bovine articular chondrocytes. NADA is also believed to have the ability to inhibit anandamide metabolism by

the fatty acid amide hydrolase (FAAH) (Bisogno *et al.*, 2000). Therefore addition of NADA to anandamide treatment would help to find out whether NADA modulates effects of anandamide. Effects of endocannabinoids, N-AG; the CB₁ cannabinoid receptor specific agonist, ACEA; and the CB₂ cannabinoid receptor specific agonist, JWH-133 were also studied on the IL-1-induced production of NO in chondrocytes.

Anandamide had no effect on IL-1-induced NO production in bovine chondrocytes (Fig. 4.5). NADA however appeared to reduce this production of NO significantly. Addition of NADA to anandamide treatments appeared not to make any significant difference (Fig. 4.5).

ACEA at all concentrations tested did not show any significant effects on NO production in articular chondrocytes (Fig. 4.6). However, N-AG from 10 μ M appeared to significantly reduce NO production (Fig. 4.6). JWH-133 on the contrary, appeared to significantly increase NO production from 30 μ M in what appeared to be a concentration dependent manner (Fig. 4.6).

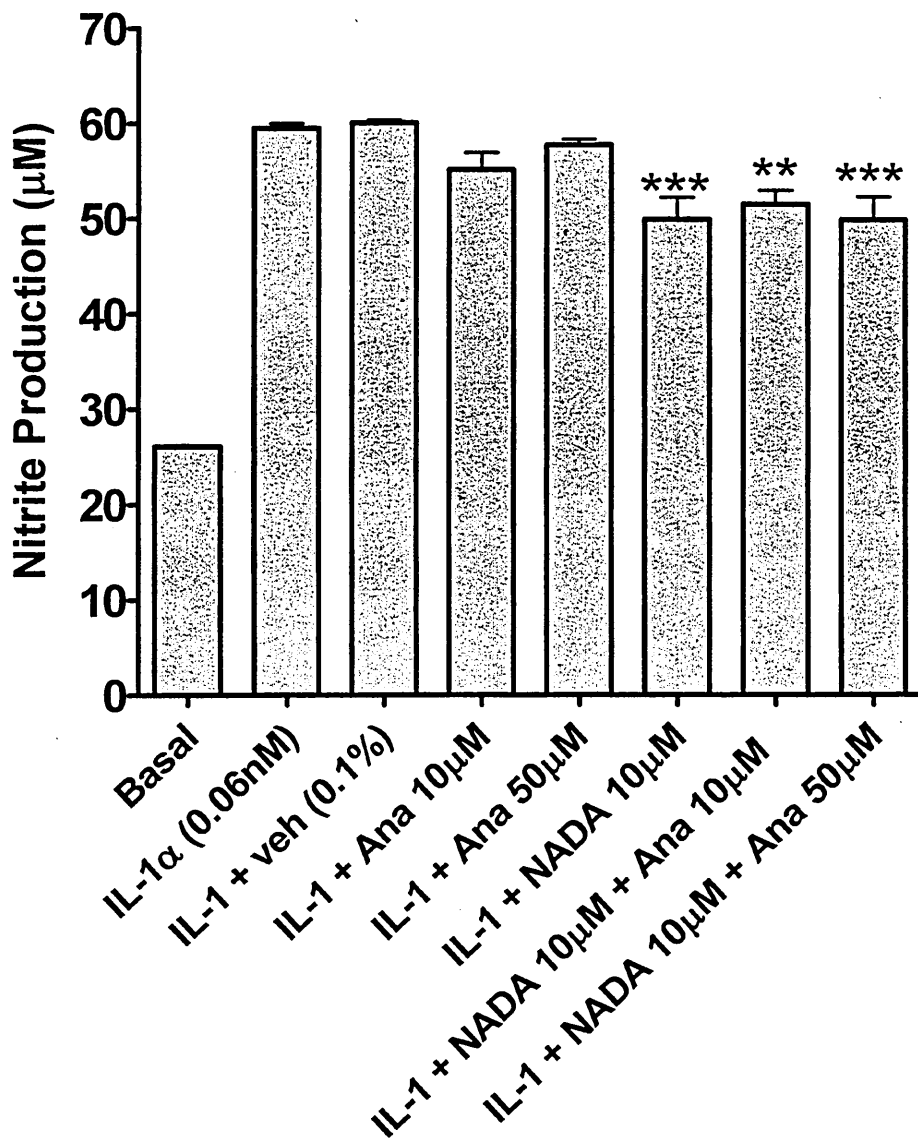


Figure 4.5: The effect of anandamide, NADA and combination of the two endocannabinoids on IL-1 α -induced NO production in bovine articular chondrocytes. Results presented as mean \pm s.e.m. (n=6). **p<0.01; ***p<0.001 compared with vehicle controls. ANOVA followed by Boniferroni's post-hoc test was used to determine statistical significance.

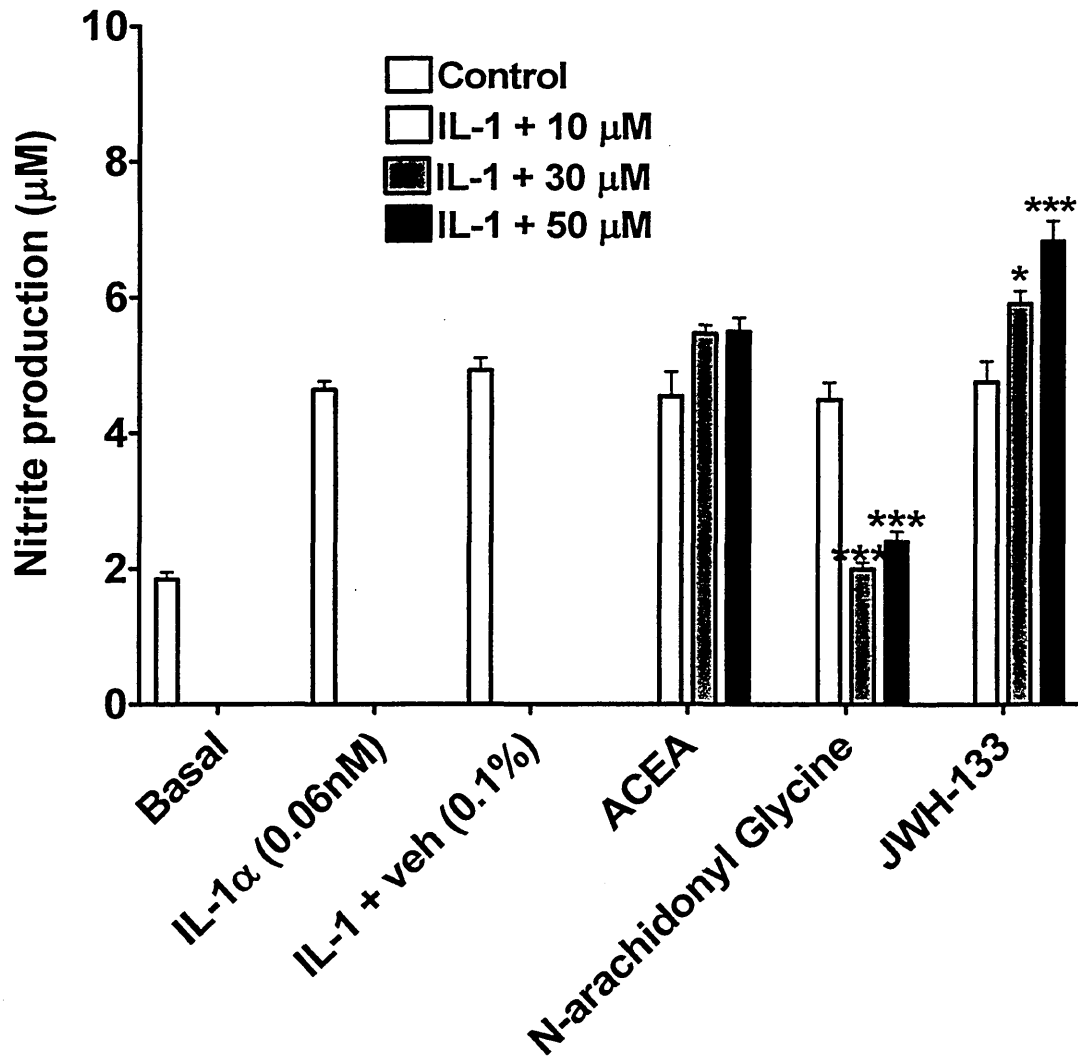


Figure 4.6: The Effect of ACEA, N-AG and JWH-133 (10-50 µM) on IL-1α-induced NO production in bovine articular chondrocytes. The results are presented as mean ± s.e.m. (n=6). *p<0.05; ***p<0.001 compared with vehicle control (ethanol). ANOVA followed by Boniferroni's post-hoc test was used to determine statistical significance

4.3.6 Effects of Win-55,212 on IL-1-stimulated iNOS expression in chondrocytes

As mentioned earlier under section 4.1, iNOS is the enzyme responsible for the increased NO production in osteoarthritic tissue and its expression is induced by IL-1 in articular chondrocytes. Therefore bovine articular

chondrocytes were stimulated with IL-1 in the presence of Win-55,212-2 to find out whether cannabinoids have the capacity to block the cytokine-induced expression of iNOS. The enzyme is approximately 131 kDa (Alderton *et al*, 2001). Equal amounts of the sample were loaded, 30 µg per well with protein content of approximately 10 µg.

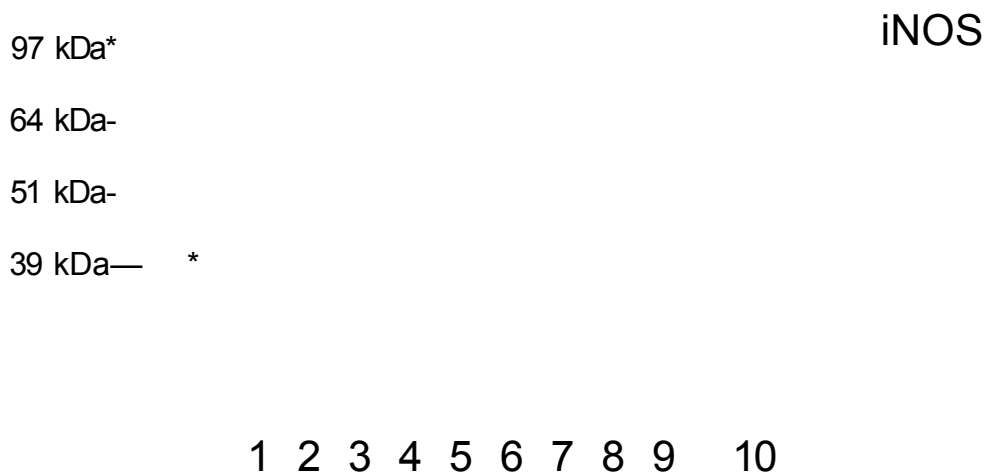


Figure 4.7: Immunoblot analysis of iNOS expression in bovine articular chondrocytes. The cells were unstimulated or stimulated with IL-1a (0.06 nM) for 48 hrs in the presence or absence of Win-55,212-2. Lane 1 & 2: Marker; Lane 3: Basal; Lane 4: IL-1a; Lane 5 & 6: vehicle control (IL-1 +0.1 % DMSO); Lane 7 & 8: IL-1 + Win-55,212-2 (1 pM); Lane 9 & 10: IL-1 + Win-55,212-2 (5 pM).

Two bands appeared on the PVDF membrane at the end of the immunoblot assay (Fig. 4.7). The first band which was faint was close to the 191 kDa, whilst the other, the main band, stained more strongly and was above 97 kDa. Its position appeared to be consistent with the reported size of iNOS, which is about 131 kDa. The first band could be iNOS bound to other proteins or undenatured iNOS which migrated more slowly than the fully denatured iNOS. It is possible also to be just a product of non-specific binding, which was also appeared to be induced by IL-1.

Expression of iNOS in this study appeared to be absent at basal levels and was significantly stimulated by IL-1 (Fig. 4.7; lanes 3 and 4, respectively). The vehicle (DMSO) appeared to slightly reduce iNOS expression (Fig. 4.7; lanes 5 & 6). Win-55,212-2 at 1 μ M did not have any effect (Fig. 4.7; lanes 7 & 8) whilst at 5 μ M blocked the IL-1-induced expression of iNOS completely (Fig. 4.7; lanes 9 & 10).

4.3.7 Effects of inhibitors MK-886, NS-398, ODQ, PDTC compared with Win-55,212-2 on IL-1-induced NO production in bovine articular chondrocytes

MK-886 is the inhibitor of 5-LOX activating protein (FLAP), the enzyme that subsequently is responsible for LTB₄ production. Its effect on NO production was studied to find out if 5-LOX activity affects NO production in chondrocytes and to compare the effects of MK-886 with those of Win-55,212-2 and if its combination with Win-55,212-2 would affect the cannabinoid's effect. The same was done for NS-398, the selective inhibitor of COX-2 activity; ODQ, the inhibitor of NO-sensitive GC; and PDTC, the inhibitor of NF- κ B that subsequently inhibits iNOS mRNA expression. The aim was to try and identify the signal transduction pathways involved in the cytokine-induced NO production and determine whether Win-55, 212-2 affected any of those pathways to produce the effect it displayed.

MK-886 reduced NO production significantly but did not augment the effect of Win-55,212-2 on NO production. NS-398 and ODQ did not show any effects. PDTC significantly reduced the production of NO and did not significantly affect the effect of Win-55,212-2 (Fig. 4.8).

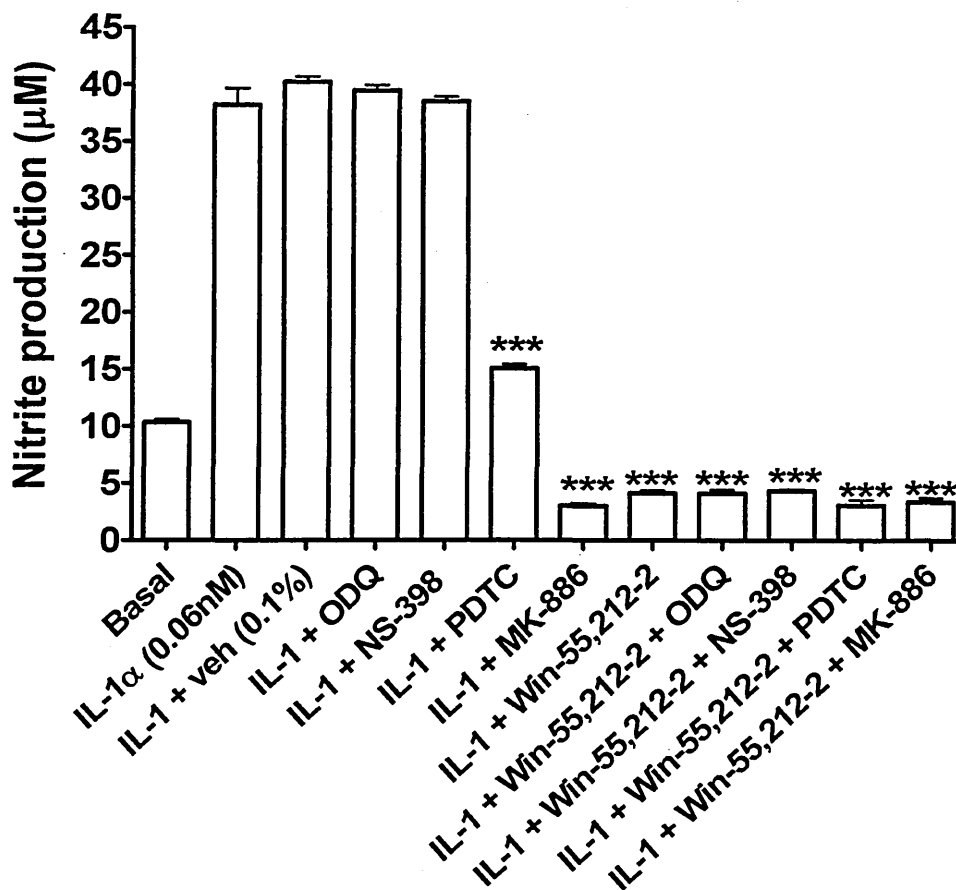


Figure 4.8: Effect of inhibitors (a) ODQ (100 µM); NS-398 (1 µM); PDTC (1 µM); MK-886 (1 µM); and Win-55,212-2 (1 µM) and combinations of these compounds on IL-1α-induced NO production in primary bovine articular chondrocyte culture. Results presented as mean ± s.e.m. (n=6). ***p<0.001 compared with vehicle controls. ANOVA followed by Boniferroni's post-hoc test was used to determine statistical significance

4.4 Discussion

Synthetic cannabinoids HU-210 and Win-55,212-2 as well as endocannabinoids NADA and N-AG appeared to exhibit anti-inflammatory effects because they significantly reduced or blocked completely IL-1-induced NO production in bovine articular chondrocytes. However it is not certain whether this is a true reflection of the effect of HU-210 on IL-1-induced NO production since HU-210 appeared to be cytotoxic at 10 µM. Win-55,212-2 also blocked the IL-1-induced expression of iNOS. Thus its reduction of NO

production in chondrocytes may be at least in part through its ability to block iNOS expression.

The inverse agonists, AM 281 and AM 630, showed some unexpected effects on NO production in bovine articular chondrocytes. When applied alone, the inverse agonists 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-55,212-2 on NO production they appeared to act synergistically when applied in combination with the cannabinoid. This implies that AM 281 and AM 630, in this case were not acting through the respective CB₁ or CB₂ cannabinoid receptors. However, AM 630 besides being an inverse agonist at CB₂ receptors also behaves as a weak partial agonist at CB₁ receptors (Ross *et al.*, 2001). Therefore it is possible that AM 630 in this study was also acting as a partial agonist at the CB₁ 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-55,212-2 was mediated through either CB₁ or CB₂ cannabinoid receptors. However the study where Win-55,212-3, the inactive cannabinoid enantiomer, was compared with the active enantiomer, Win-55,212-2, suggested otherwise (section 4.3.3). Win-55,212-3 did not show any significant effects whilst Win-55,212-2 significantly blocked the IL-1-induced NO production in chondrocytes. This stereo-specificity indicates that Win-55,212-2 produced its effects by acting through some receptor, possibly CB₁, CB₂ or an additional receptor yet to be identified.

NADA did not modulate the effects of anandamide, implying that lack of effect by anandamide may be due its low potency not its metabolism by FAAH. It is possible also that the concentration of NADA used was too low thus it did not work efficiently to protect anandamide in this system.

Inhibitors MK-886, inhibitor of FLAP and PDTC, inhibitor of NF- κ B significantly reduced the NO production induced by IL-1. MK-886 reduced it to the level

comparable to the effect of Win-55,212-2 and it did not affect the activity of Win-55,212-2 when applied in combination. The results suggest that 5-LOX activation may enhance NO production induced by IL-1 and it is possible that Win-55,212-2 may be acting in part as this inhibitor, blocking the 5-LOX pathway. PDTC, although it significantly reduced the production of NO, did not reduce it to basal levels as Win-55,212-2 did. The transcription factor, NF- κ B is essential for activation of numerous genes for proteins involved in inflammation such as pro-inflammatory proteins; iNOS may be one of them. PDTC is capable of inhibiting expression of iNOS mRNA and this may be as a consequence of inhibition of NF- κ B. Inhibition of iNOS results in reduced production of NO and this may explain the observed effects of PDTC. PDTC did not affect the effect of Win-55,212-2 and it is possible that the latter reduced NO production in part by acting as PDTC, inhibiting activation of NF- κ B.

NO production by chondrocytes stimulated by IL-1 may be one of mechanisms that promote the breakdown of cartilage. In cytokine-stimulated chondrocytes, NO sustains nuclear translocation of NF- κ B and thus keeps NF- κ B-dependent transcription persistently switched on (Clancy *et al.*, 2004). 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 since NO plays a regulatory role in the activation of MMPs in articular chondrocytes and cartilage (Murrell *et al.*, 1995). This is supported by the report that selective inhibition of NF- κ B blocks inflammatory bone destruction (Jimi *et al.*, 2004).

NO production in early phases of arthritis, *in vivo*, may lead to chondrocyte apoptosis (Taskiran *et al.*, 1994). Tawara *et al.* (1991) also report that IL-1 significantly enhanced production of superoxides in chondrocytes. NO, in the presence of superoxide anions, was shown to induce chondrocyte apoptosis (Mathy-Hartert *et al.*, 2005). Apoptotic death of articular chondrocytes contributes to articular cartilage degradation (Goggs *et al.*, 2003).

Endogenously synthesized NO also suppresses proteoglycan synthesis in articular chondrocytes (Häuselmann et al., 1994) and (Taskiran *et al.*, 1994). Also IL-1-induced production of NO partially inhibits synthesis of type II collagen. This NO-dependent inhibition may occur at the post-translational level, where collagen is subject to several processing stages including hydroxylation by prolyl hydroxylase (Goggs *et al.*, 2003). Inhibition of NO synthase was shown to strongly suppress the production of NO and partially relieve the inhibition of collagen synthesis in response to IL-1 whilst NO donors inhibited collagen production (Cao et al., 1997). The studies by Cao et al. (1997) suggested that inhibition of prolyl hydroxylase by NO might be responsible for the suppressive effect of this radical molecule on collagen synthesis since under-hydroxylated collagen monomers fail to anneal into stable triple helices and are degraded intracellularly. 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 and maintaining the integrity of the cartilage.

NO is capable of inducing the inflammatory component of OA as well as tissue destruction. Therefore it could be responsible not only for the symptoms, but also for the disease process and thus is a most interesting target (Pelletier et al., 1998). In the same way, therefore, cannabinoids may have a potential as therapeutic agent in arthritic diseases; by reducing excess NO production, cannabinoids may be capable of reducing not only the symptoms but also the progression of disease, thus concurrently reaching two targets.

Conclusion

Cannabinoids have the capacity to inhibit IL-1 α -induced NO production at micromolar concentrations in bovine articular chondrocytes. Win-55,212-2 appeared to be the most potent compared with the rest of the cannabinoids tested. The attenuation of NO production may be partly through the capacity of the cannabinoids to block iNOS expression.

CHAPTER 5

CANNABINOIDS AND THEIR EFFECT ON EICOSANOIDS PRODUCTION IN ARTICULAR CHONDROCYTES

5.1 Introduction

Eicosanoids play a significant role not only in joint physiology, but also in the pathogenesis of joint disorders (Laufer, 2003). In response to IL-1, chondrocytes upregulate the production of PGE₂, one of the factors that have been shown to induce a number of the cellular changes associated with OA (Hedbom and Hauselmann, 2002). The superinduction of PGE₂ production coincides with the upregulation of cyclooxygenase-2 (COX-2) in OA-affected cartilage (Amin et al., 1997). PGE₂ derived from COX-2 modulates cartilage proteoglycan degradation in human osteoarthritis explants (Hardy *et al.*, 2002). In addition, it has been identified that osteoarthritis subchondral osteoblasts can synthesize LTB₄, indicating a role of leukotrienes in bone remodeling associated with osteoarthritis (Laufer, 2003). LTB₄ probably contributes to the up-regulation of important catabolic factors involved in the pathophysiology of OA, such as MMP (Martel-Pelletier et al., 2004). A therapeutic intervention that blocks lipoxygenase/cyclooxygenase pathways, thereby inhibiting production of prostaglandins and leukotrienes, may therefore be very attractive for the treatment of OA (Laufer, 2003). Cannabinoid effects on IL-1-induced PGE₂ and LTB₄ production in bovine primary articular chondrocytes were investigated.

5.2 Materials and Methods

5.2.1 Cannabinoids and drugs: N-Arachidonyl glycine (N-AG); the synthetic cannabinoids, ACEA, Win-55,212-2, HU-210 and JWH-133; and the antagonists, AM281 and AM630 which are also inverse agonists of cannabinoid receptors CB₁ and CB₂ respectively, were studied along with their solvent controls. Absolute ethanol (0.1% final concentration) and dimethyl sulfoxide (DMSO) (0.1% final concentration) were used as the solvent according to instructions from the suppliers of the cannabinoids. Different inhibitors were also studied, namely: MK-886, inhibitor of FLAP; NS 398, inhibitor of cyclooxygenase 2 (COX-2); ODQ, inhibitor of NO-sensitive guanylyl cyclase; and pyrrolidine dithiocarbamate ammonium (PDTCA), inhibitor of NF-κB. Their effects were investigated on unstimulated or IL-1α-

stimulated chondrocyte PGE₂ and LTB₄ production and COX-1, COX-2 and mPGES-2 expression at concentrations which were demonstrated to be non-toxic to chondrocytes.

5.2.2 Bovine chondrocyte culture: Bovine articular chondrocytes were cultured as described in section 2.2.1. When the chondrocytes reached near confluence (about 7-10 days) they were stimulated with IL-1 α (100 U/ml \equiv 0.06 nM) (Buttle *et al.*, 1997) and were incubated with the drugs for 48 h in DMEM with the supplements except FBS.

5.2.3 PGE₂ and LTB₄ determination: After 48 hrs incubation of the chondrocytes with the drugs or inhibitors, PGE₂ and LTB₄ production was measured using an ELISA (Alexis) according to the manufacturer's instructions, refer to section 2.2.5.

5.2.4 Immunoblotting: rabbit polyclonal COX-1 and COX-2 and mPGES-2 (diluted 1:100) (Alexis Biochemical) were used. The rest of the immunoblot analysis was carried out as described under section 2.2.7.

5.2.5 Immunofluorescence: Polyclonal COX-2 (1:50) (Alexis Biochemical) was used to study expression of COX-2 in chondrocytes and the effect of Win-55,212-2 on this was determined. The immunofluorescence studies were carried out as described in section 2.2.9

5.3 Results

5.3.1 Effect of Win-55,212-2 on PGE₂ production in chondrocytes

Chondrocytes were stimulated with IL-1 α (0.06nM) to produce PGE₂. The cells were incubated with Win-55,212-2 at 1-10 μ M for 48 hrs and PGE₂ production was measured. The effect of Win-55,212-2 was compared with its vehicle (DMSO, 0.1% final concentration)

Win-55,212-2 did not show any effects at 1 μ M but significantly ($p < 0.01$) reduced IL-1-induced PGE₂ production at 5 and 10 μ M, compared to the vehicle control in bovine chondrocyte cultures (Fig. 5.1).

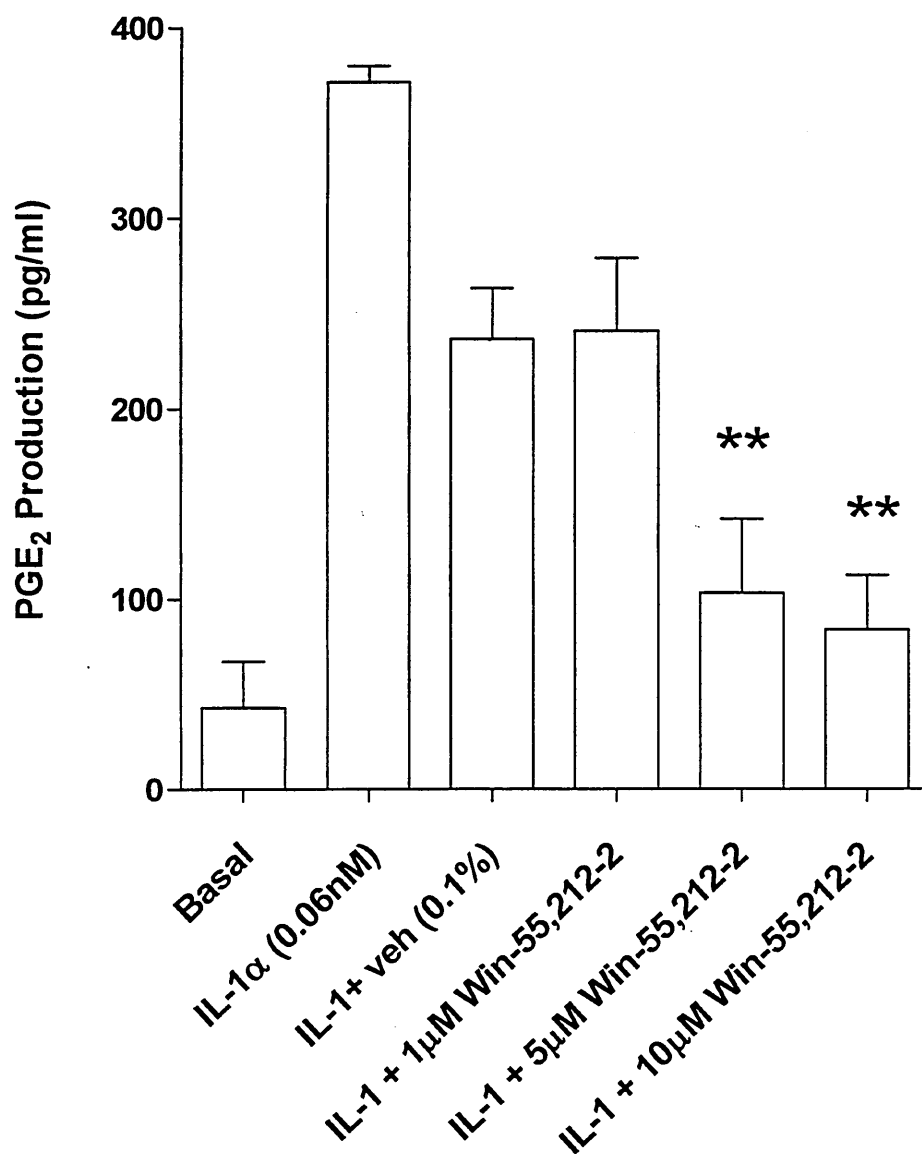


Figure 5.1: Effect of R-(+)-Win-55,212 on IL-1 α -induced PGE₂ production in primary bovine articular chondrocyte culture. The results are presented as mean \pm s.e.m. (n=6). ** $p < 0.01$ compared with vehicle control. ANOVA followed by Boniferroni's post-hoc test were used to determine statistical significance.

5.3.2 Effect of HU-210 on PGE₂ production in bovine chondrocytes

HU-210, a non-selective cannabinoid receptor agonist, was studied to find out its effect on the IL-1 α -induced PGE₂ production in bovine articular chondrocytes incubated for 48 hrs.

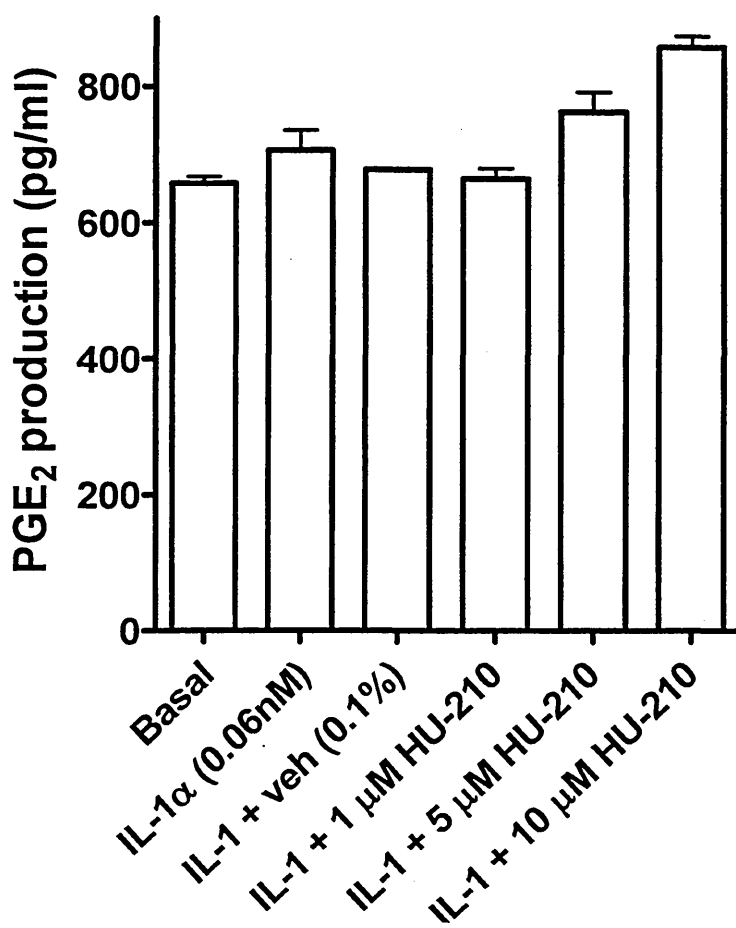


Figure 5.2: Effect of HU-210 on IL-1 α -induced PGE₂ production in primary bovine articular chondrocyte culture. Results presented as mean \pm SD. The treatments were run in duplicates and the experiment was performed once.

The cells in this experiment (Fig. 5.2) appeared to have high basal level of PGE₂ and IL-1 slightly induced PGE₂ production in these cells. Nonetheless, HU-210 appeared to enhance PGE₂ production in a concentration-dependent manner.

5.3.3 Effect of ACEA, N-AG and JWH-133 on PGE₂ production in chondrocytes

The endocannabinoids, N-AG as well as the synthetic ACEA CB₁ cannabinoid receptor specific agonist and CB₂ cannabinoid receptor specific agonist JWH-133 were studied to determine their effects on IL-1 α induced PGE₂ production in chondrocytes. The chondrocytes were stimulated with IL-1 and incubated in the presence of the cannabinoids for 48 hrs.

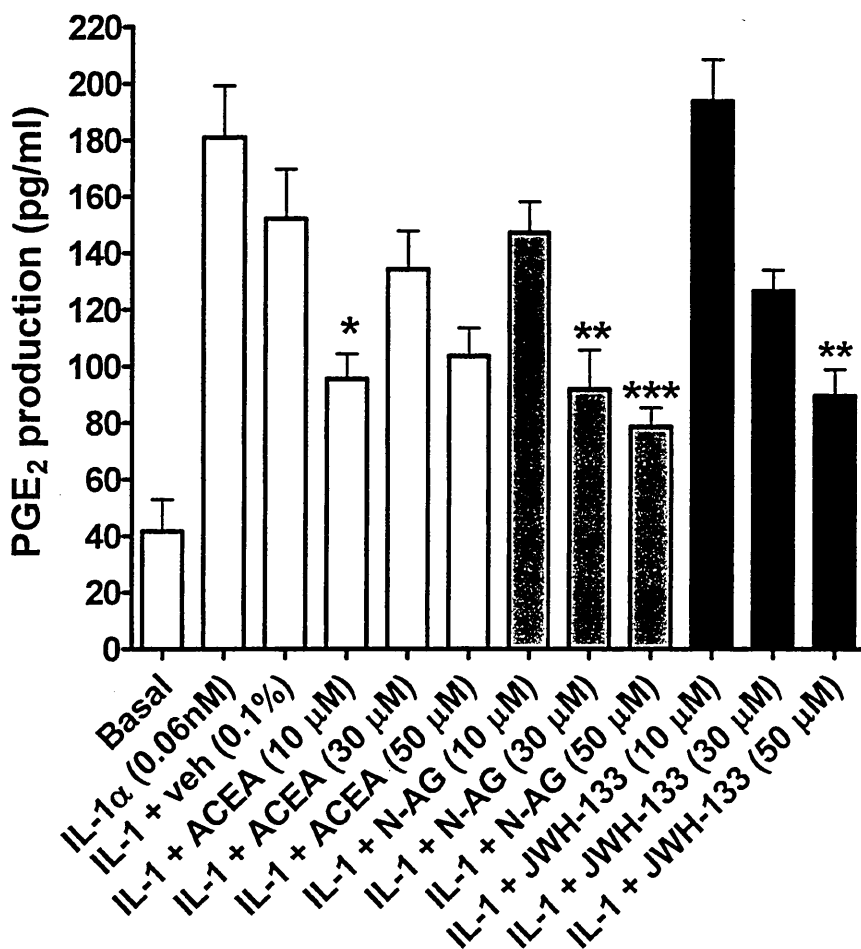


Figure 5.3: Effects of ACEA, N-AG and JWH-133 at 10, 30 and 50 μ M on IL-1 α -induced PGE₂ production in primary bovine articular chondrocyte culture. Results presented as mean \pm s.e.m (n=6). *p<0.05; **p<0.01; ***p<0.001; compared with vehicle control (ethanol). ANOVA followed by Boniferroni's post-hoc test were used to determine statistical significance.

ACEA significantly ($p < 0.05$) reduced PGE₂ production in bovine articular chondrocytes at 10 μ M, did not show significant effect at 30 and 50 μ M, albeit reduced PGE₂ slightly (Fig. 5.3). N-AG apparently reduced PGE₂ production at 30 and 50 μ M in a concentration dependent manner in this system (Fig. 5.3). JWH-133 appeared to slightly enhance PGE₂ production at 10 μ M, and slightly reduced its production at 30 μ M and significantly reduced PGE₂ production at 50 μ M (Fig. 5.3). This effect of JWH-133 also appears to be concentration dependent.

5.3.4 Effects of different inhibitors on PGE₂ production in articular chondrocytes

MK-886, the inhibitor of 5-LOX activating protein (FLAP); NS-398, inhibitor of COX-2; ODQ, the inhibitor of NO-sensitive GC; and PDTC, the inhibitor of NF- κ B were studied to determine their effects on the production of PGE₂ induced by IL-1 in bovine articular chondrocytes. The cells were treated for 48 hrs. The aim was to find out the pathways involved in IL-1-induced PGE₂ production in chondrocytes.

All the inhibitors blocked the IL-1-induced production of PGE₂ in chondrocytes, except for MK-886 which did not show any significant effects (Fig 5.4 and 5.5). MK-886 appeared to slightly block the inhibition of PGE₂ production by Win-55,212-2 (Fig. 5.5). When applied to the cells in combination with NS-398, MK-886 appeared not to affect the blockage of PGE₂ production by NS-398, which appeared to be more potent, compared with Win-55,212-2 (Fig. 5.5). Addition of MK-886 and Win-55,212-2 to the NS-398 treatments did not alter its effects (Fig. 5.5). ODQ also blocked PGE₂ production in these cells and so did PDTC (Fig. 5.4 and 5.5).

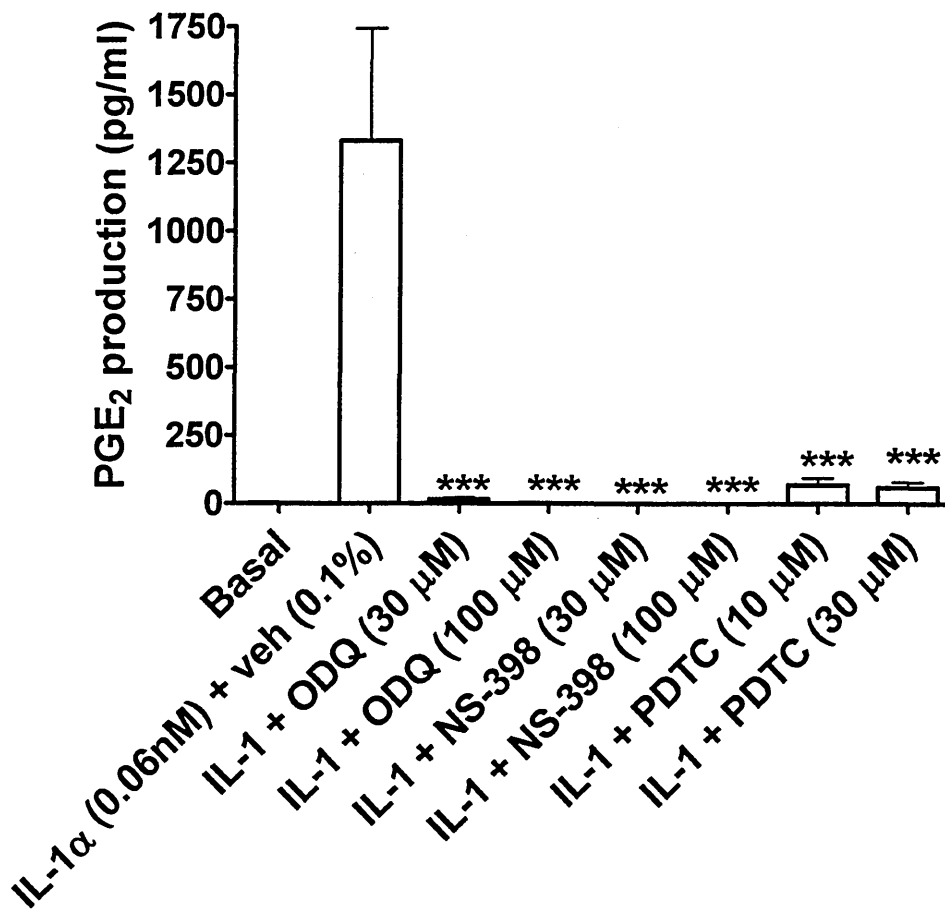


Figure 5.4: Effect of inhibitors (a) ODQ (30 and 100 μ M); NS-398 (30 and 100 μ M) and PDTC (10 and 30 μ M) on IL-1 α -induced PGE₂ production in primary bovine articular chondrocyte culture. The results are presented as mean \pm s.e.m (n=6). ***p<0.001 compared with vehicle controls. ANOVA followed by Boniferroni's post-hoc test were used to determine statistical significance.

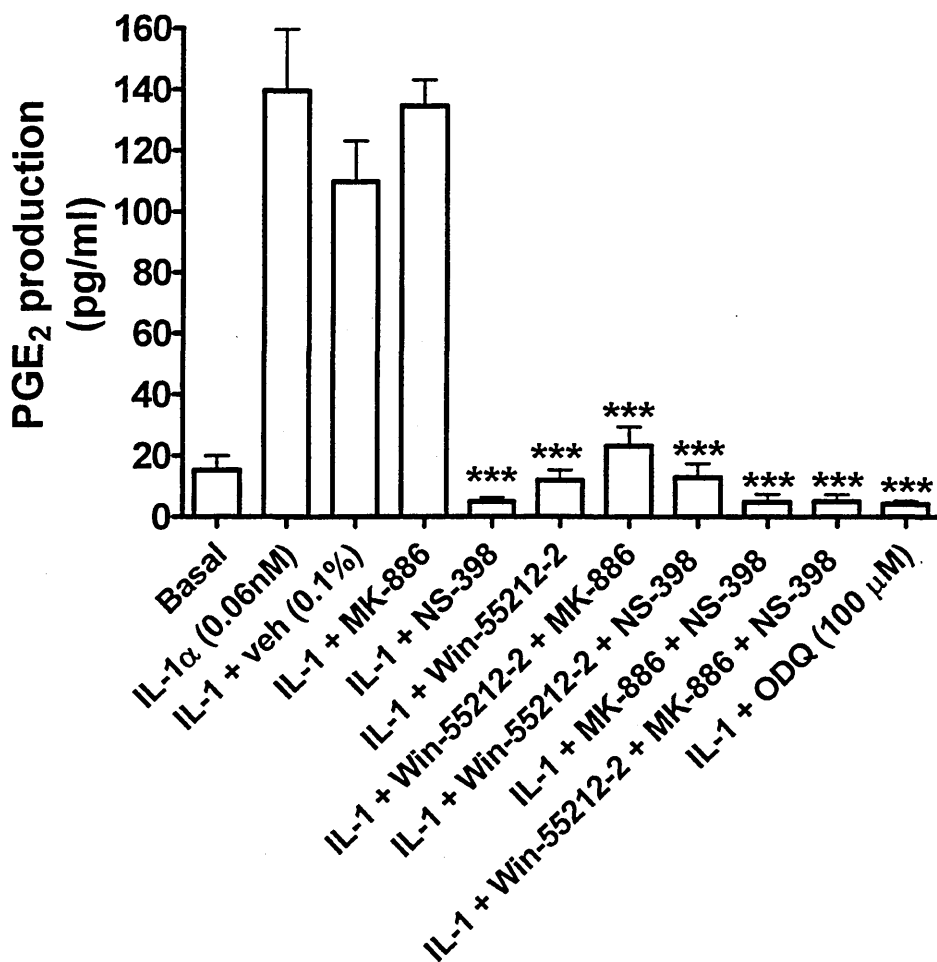


Figure 5.5: Effect of inhibitors MK-886 (1 μM); NS-398 (1 μM); Win-55,212-2 (1μM), ODQ (100 μM) and combinations of these compounds on IL-1α-induced PGE₂ production in primary bovine articular chondrocyte culture. The results are presented as mean ± s.e.m (n=6). ***p<0.001 compared with vehicle controls. ANOVA followed by Boniferroni's post-hoc test were used to determine statistical significance.

5.3.5 Effects of Win-55,212-2 on IL-1-induced COX-1, COX-2 and mPGES-2 expression in bovine articular chondrocytes.

Expression of enzymes, COX-1, COX-2 and microsomal PGE₂ synthase-2 (mPGES-2) involved in the constitutive or inducible production of PGE₂ in bovine articular chondrocytes stimulated by IL-1, and the effects of Win-55,212-2 at 1 and 5 μM on the enzymes expression were studied.

5.3.5.1 Immunofluorescence: COX-2 expression

Expression of COX-2 in bovine articular chondrocytes unstimulated or stimulated with IL-1 for 48 hrs and the effect of Win-55,212-2 at 5 pM on this was studied through indirect immunofluorescence.

Figure 5.6: Immunofluorescence analysis of COX-2 expression in bovine articular chondrocytes cultured for 7 days. The cells were unstimulated (B) or stimulated with IL-1a (0.06 nM) in the presence of: 0.1 % vehicle - DMSO (C); 5 pM Win-55,212-2 (D). Negative control (A) (NGS used instead of primary antibody). Primary antibody against COX-2 was diluted 1:50 and FITC-conjugated secondary antibody was used to visualise COX-2 expression.

In this experiment, the expression of COX-2, the inducible enzyme which produces PGE₂, was not expected in the basal treatment but the chondrocytes appeared to have been stimulated in one way or the other (Fig. 5.6 B). IL-1 appeared to enhance the expression further (Fig. 5.6 C). Win-55,212-2 at 5 pM blocked the expression of COX-2 in the cells (Fig. 5.6 D).

S.3.5.2 immunoblot analysis on cyclooxygenase (COX) expression

Expression of COX-2 in bovine articular chondrocytes was also studied through immunoblotting. Its expression was compared to the expression of COX-1 in these cells. COXs have their molecular weight around 64 kDa. The molecular weight of the enzymes depends on whether it is glycosylated or not (Percival *et al.*, 1994).



Figure 5.7: Immunoblot analysis of COX-1 and COX-2 expression in bovine articular chondrocytes. The cells were unstimulated or stimulated with IL-1a (0.06 nM) for 48 hrs in the presence or absence of Win-55,212-2. Lane 1: Marker; Lane 2: Basal; Lane 3 & 4: IL-1a; Lane 5 & 6: vehicle control (IL-1 + 0.1 % DMSO); Lane 7 & 8: IL-1 + Win-55,212-2 (1 pM); Lane 9 & 10: IL-1 + Win-55,212-2 (5 pM).

The results indicated that expression of COX-2 was faint at 48 hrs following IL-1 stimulation in this system. This was not surprising because COX-2 expression is time-dependent; it is maximal at 24 hrs (Kojima *et al.*, 2004; Nedelec *et al.*, 2001). Low levels, nonetheless, are still expressed at 48 hrs following stimulation. In the COX-2 blot (Fig. 5.7), the membrane was incubated for 2 hrs in substrate in an attempt to amplify the signal further. The bands appear to be where they would be expected with the COX-2 band appearing to be slightly above 64 kDa and the COX-1 band at 64 kDa. The blot shows no basal expression of COX-2 (Fig. 5.7, lane 2). IL-1 induced its expression. Win-55,212-2 at 1 pM (Fig. 5.7, lanes 7&8) did not significantly

affect IL-1-induced COX-2 expression compared with the vehicle control (Fig. 5.7, lanes 5&6). However, Win-55,212-2 at 5 pM blocked the IL-1-induced expression of COX-2 (Fig. 5.7, lanes 9&10). COX-1 expression appeared not to be affected by IL-1 or Win-55,212-2. It was constitutively expressed (Fig. 5.7)

5.3.5.3 immunoblot analysis: mPGES-2 expression in chondrocytes

Whole cell lysates from chondrocytes unstimulated or stimulated by IL-1 and incubated with or without Win-55,212-2, were analysed by immunoblotting for expression of one of the enzymes in the final step in the biosynthesis of PGE₂ - mPGES-2. The molecular weight of mPGES-2 is approximately 60 kDa, consisting of a homo-dimer of approximately 30 kDa subunits (Wanatabe *et al.*, 1999).

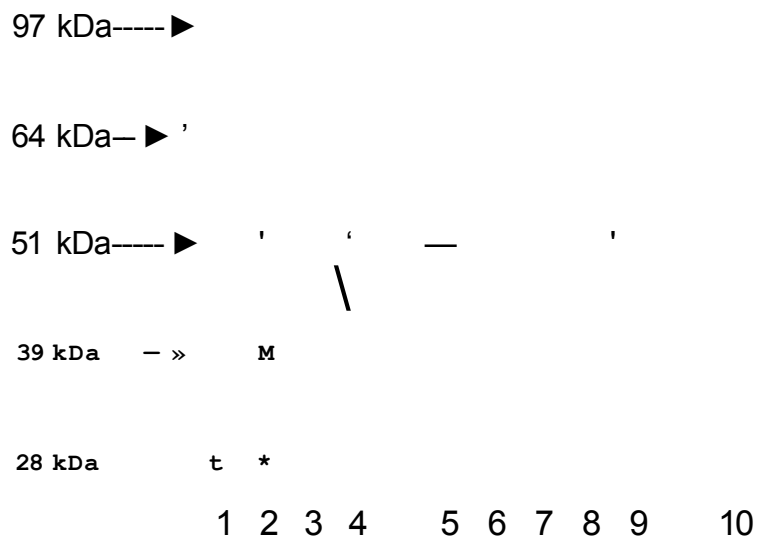


Figure 5.8: Immunoblot analysis of microsomal PGE₂ synthase-2 (mPGES-2) expression in bovine articular chondrocytes using rabbit anti-mPGES-2 antibody. The cells were unstimulated or stimulated with IL-1a (0.06 nM) for 48 hrs in the presence or absence of Win-55,212-2. Lane 1 & 2: Marker; Lane 3: Basal; Lane 4: IL-1a; Lane 5 & 6: vehicle control (IL-1 + 0.1 % DMSO);

Lane 7 & 8: IL-1 + Win-55,212-2 (1 μ M); Lane 9 & 10: IL-1 + Win-55,212-2 (5 μ M).

Three bands were visualised, one approximately 80 kDa, the second one appeared between 51 and 64 kDa and last one (the main band) was between 28 and 39 kDa (Fig. 5.8). The band between 51 and 64 kDa possibly represented the homo-dimer, whilst the band below it (the main band), represented the protein subunit. It is not certain what the approximately 80 kDa band represents. The mPGES-2 was expressed constitutively and was not affected by IL-1 or the drugs except at 5 μ M Win-55,212-2 where the expression appeared to be reduced slightly (Fig. 5.8, lanes 9&10).

5.3.6 Effects of cannabinoids on LTB₄ production in chondrocytes

Bovine articular chondrocytes were stimulated with IL-1 in the presence of cannabinoids and/or inhibitors. Production of LTB₄ in these cells was measured and effects of the drugs were studied.

5.3.6.1 Effect of Win-55,212-2 and HU-210 on LTB₄ production

Effects of non-selective cannabinoid receptors, HU-210 and Win-55,212-2 on IL-1 α (0.06nM) induced production of LTB₄ in bovine articular chondrocytes. The cells appeared to constitutively produce LTB₄ whilst IL-1 α appeared to reduce LTB₄ production in these cells instead of stimulating its production (Fig. 5.9 and 5.10). These findings are not unprecedented, Tawara *et al.* (1991) also showed that chondrocytes spontaneously released LTB₄ into culture medium and IL-1 β significantly inhibited LTB₄ production by these cells.

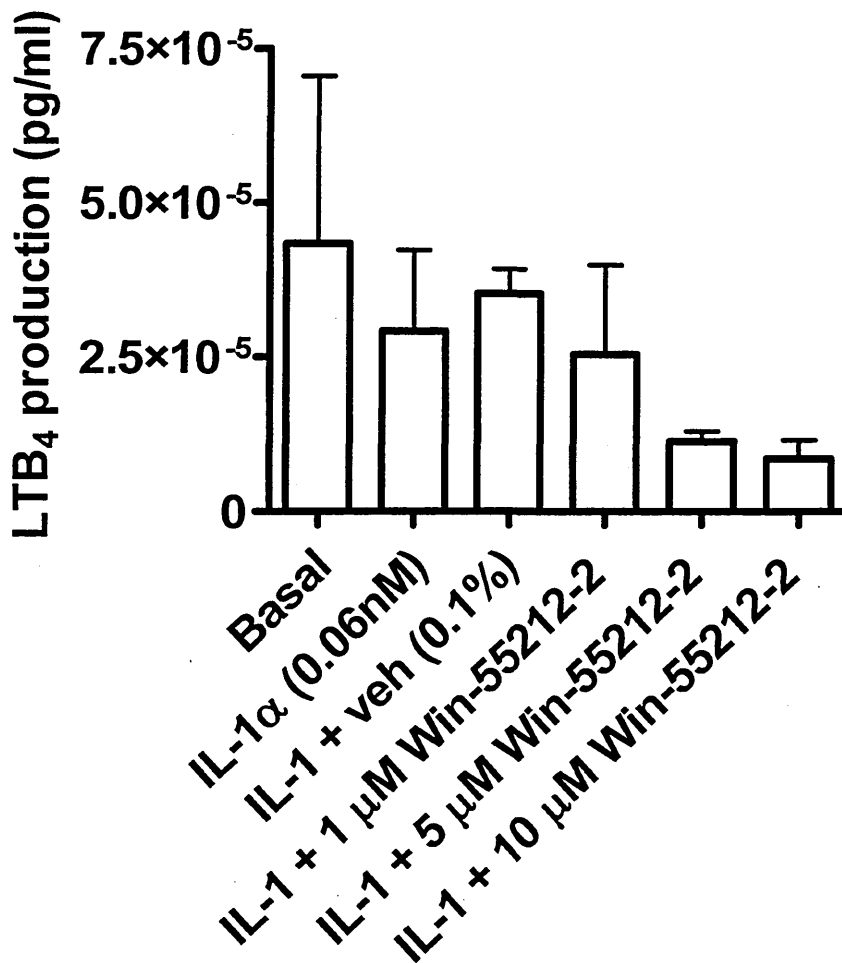


Figure 5.9: Effect of Win-55,212-2 on IL-1 α -induced LTB₄ production in primary bovine articular chondrocyte culture. Results presented as mean \pm SD (n=3).

HU-210 at high concentration (10 μ M) significantly increased LTB₄ production in chondrocytes and appeared to attenuate the production of LTB₄ at low concentration (1 μ M) (Fig. 5.10). Win-55,212-2 on the other hand appeared to reduce LTB₄ production in a concentration-dependent manner (Fig. 5.9).

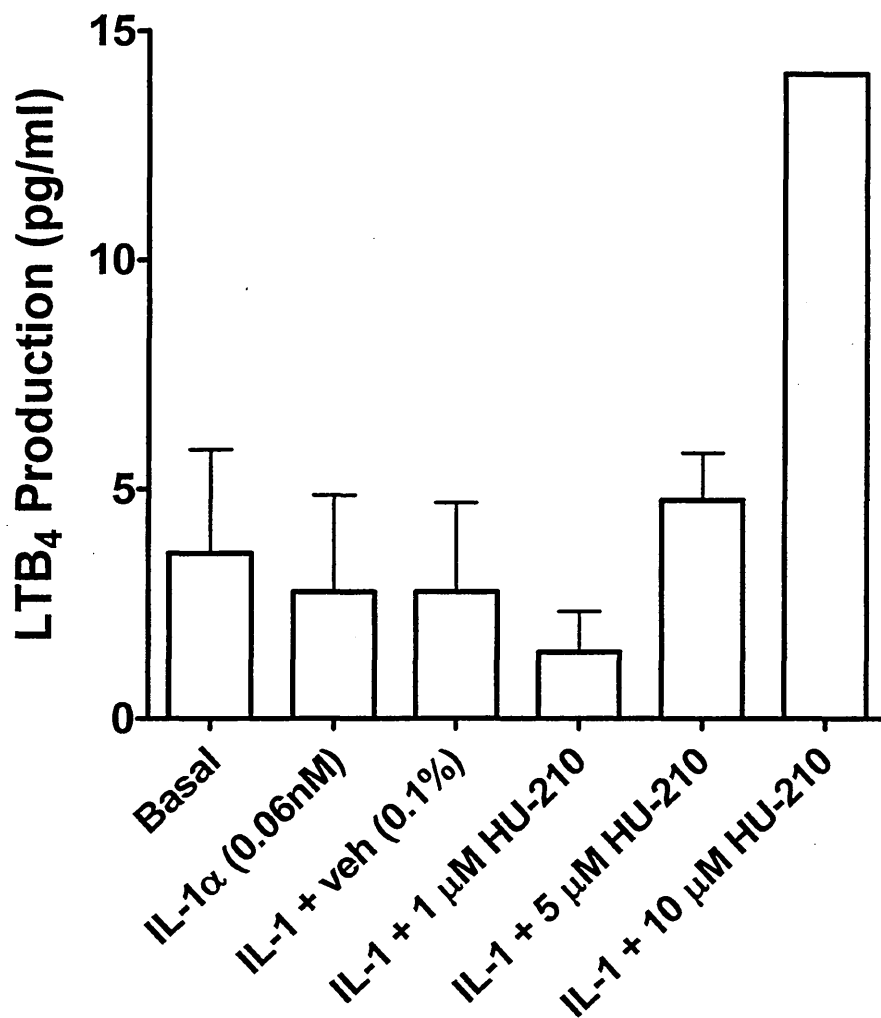


Figure 5.10: Effect of HU-210 (1, 5 and 10 μM) on IL-1 α -induced LTB₄ production in primary bovine articular chondrocyte culture. Results presented as mean \pm SD (n=3).

5.3.6.2 Effect of cannabinoids ACEA, N-AG and JWH-133 on LTB₄ production

LTB₄ production in IL-1 stimulated bovine articular chondrocytes was studied in the presence of endocannabinoid, N-AG; CB₁ cannabinoid receptor specific, ACEA; and CB₂ cannabinoid receptor specific, JWH-133.

ACEA and N-AG appeared to augment production of LTB₄ in chondrocytes in a concentration-dependent manner (Table 5.1). N-AG significantly augmented LTB₄ production.

Treatment	Average (pg/ml)	SD
Basal	0.001	0.0004
IL-1 α (0.06nM)	0.0004	0.0002
IL-1 + Vehicle	0.001	0.001
IL-1 + ACEA (10 μ M)	0.001	0.001
IL-1 + ACEA (30 μ M)	0.002	0.001
IL-1 + ACEA (50 μ M)	0.012	0.008
IL-1 + N-AG (10 μ M)	0.011	0.006
IL-1 + N-AG (30 μ M)	0.256	0.320
IL-1 + N-AG (50 μ M)	9.809	4.353
IL-1 + JWH-133 (10 μ M)	0.005	0.003
IL-1 + JWH-133 (30 μ M)	0.004	0.002
IL-1 + JWH-133 (50 μ M)	0.002	0.001

Table 5.1: Effect of ACEA, N-AG and JWH-133 (10, 30 and 50 μ M) on IL-1 α -induced LTB₄ production in primary bovine articular chondrocyte culture. Results presented as mean \pm SD (n=6).

5.3.6.3 Effect of ODQ, NS-398 and PDTC on LTB₄ production

The effects of NO-sensitive GC inhibitor, ODQ; inhibitor of COX-2, NS-398; and NF- κ B inhibitor, PDTC on production of LTB₄ in bovine articular chondrocytes

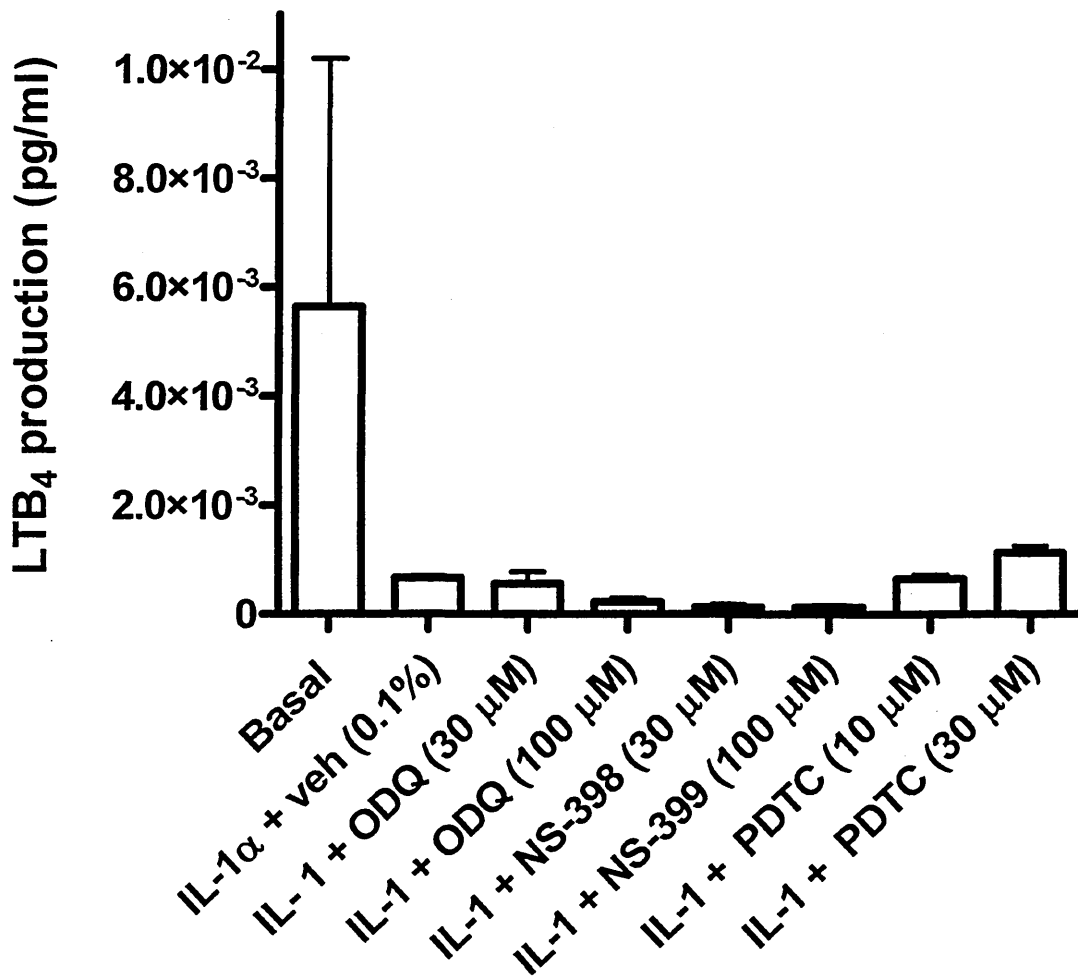


Figure 5.11: Effect of inhibitors ODQ (30 and 100 μM); NS-398 (30 and 100 μM); and PDTC (10 and 30 μM) on IL-1 α -induced LTB₄ production in primary bovine articular chondrocyte culture. Results presented as mean \pm s.e.m. (n=6).

Inhibition of NO-sensitive GC by ODQ appeared to reduce LTB₄ production in a concentration dependent-manner (Fig. 5.11). Inhibition of COX-2 by NS-398 also blocked LTB₄ production in chondrocytes (Fig. 5.11). On the contrary, inhibition of NF- κ B by PDTC appeared to enhance LTB₄ production in a concentration- dependent manner (Fig. 5.11).

5.4 Discussion

All cannabinoids tested appeared to have the capacity to reduce the IL-1-induced production of PGE₂ in bovine articular chondrocytes, except for HU-210 which appeared to augment the production in a concentration-dependent manner. Win-55,212-2 appeared to be the most potent of the cannabinoids and its effect was concentration-dependent.

Inhibition of NO-sensitive guanylate cyclase (GC) by ODQ significantly ($p < 0.001$) reduced PGE₂ production in articular chondrocytes. Suggesting that NO-sensitive GC pathway is involved in the IL-1-induced PGE₂ production in this system. COX-2 inhibition through NS-398, also significantly ($p < 0.001$) reduced PGE₂ production in these chondrocytes, at 1 μ M it reduced PGE₂ production to below basal levels. Thus IL-1-induced PGE₂ production was through COX-2 activity. Inhibition of NF- κ B by pyrrolidine dithiocarbamate (PDTC) significantly reduced IL-1 enhanced PGE₂ production in these articular chondrocytes. This suggests that NF- κ B is one of the mediators of IL-1-induced PGE₂ production in these cells.

5-LOX inhibitor, MK-886, appeared to slightly enhance the IL-1-induced PGE₂ production in articular chondrocytes. Implying that blocking the 5-LOX pathway may enhance the induction of the COX pathway. This has also been reported by Martel-Pelletier *et al.* (2004).

IL-1 α up-regulated the production of PGE₂ in articular chondrocytes and Win-55,212-2 appeared to have the ability to attenuate the production of PGE₂ by blocking COX-2 expression (Fig. 5.6-5.7). Win-55,212-2 in this study has been shown to be a specific COX-2 inhibitor since it did not affect expression of COX-1. It affected the expression of the constitutively produced mPGES-2 slightly. This implies that the physiological roles of COX-1 and mPGES-2 may not be affected by use of the drug, thus may have fewer side effects, if any, compared to other COX-2 inhibitors that also affect COX-1 expression and other constitutively produced enzymes in this pathway.

It has been reported that the induction of COX-2 expression and the subsequent increase in PGE₂ production are essential for NO-induced chondrocyte apoptosis and that selective inhibition of COX-2 completely blocks this apoptotic phenomenon (Notoya *et al.*, 2000). PGE₂ produced in chondrocytes has been thought to induce apoptosis in these cells, linked with the cAMP-dependent pathway (Miwa *et al.*, 2000) and since cannabinoids inhibit cAMP production by inhibiting adenylate cyclase (Howlett *et al.*, 2002), cannabinoids could inhibit PGE₂-dependent chondrocyte apoptosis by directly inhibiting PGE₂ production and/or by inhibiting the cAMP-dependent pathway. Since apoptotic death of articular chondrocytes has been implicated in cartilage degradation (Goggs *et al.*, 2003), its inhibition would protect the cartilage and could be one of the ways in which cannabinoids are able to protect cartilage from cytokine-induced degradation.

In studies done by Hardy *et al.* (2002), IL-1 induced cartilage proteoglycan degradation in OA synovial membrane-cartilage cocultures and this phenomenon corresponded to the induction of COX-2 protein expression and PGE₂ production in this system. IL-1 neutralising antibody or the selective COX-2 inhibition attenuated both the IL-1-induced PGE₂ production and cartilage proteoglycan degradation in these cocultures. Addition of PGE₂ reversed the inhibition of proteoglycan degradation caused by COX-2 inhibition suggesting that induction of synovial COX-2-produced PGE₂ is one mechanism by which IL-1 modulates cartilage proteoglycan degradation in OA. COX-2 inhibitors counteract some of the effects of IL-1 on human chondrocyte/cartilage metabolism. COX-2 inhibition was shown to be able to restore the proteoglycan synthetic response to TGF- β in IL-1 activated human chondrocytes. Thus these inhibitors may be used to minimize cartilage damage in arthritic and mechanically stressed joints (Studer and Chu, 2005).

High levels of PGE₂ have been shown to up-regulate the expression and synthesis of Insulin-like growth factor-1 (IGF-1) binding proteins (IGFBPs) such as IGFBP-3, -4 and -5 (Di Battista *et al.*, 1996; Sunic *et al.*, 1998). These IGFBPs sequester IGF-1 thus reducing the anabolic stimulation of this tissue and contributing to the net loss of cartilage in inflammatory and

degenerative arthritides (Niedel *et al.*, 1997; Olney *et al.*, 1996). Expression of these IGFBPs has also been reported to be elevated in osteoarthritic cartilage (Olney *et al.*, 1996) and rheumatoid arthritis synovial fluid (Niedel *et al.*, 1997). Since PGE₂ has been shown to be involved in the activation of the IGF-1/IGFBP axis, Di Battista *et al.* (1997) suggested that PGE₂ may be a rate-limiting step in cartilage repair processes. Cannabinoids, by inhibiting PGE₂ production, may be interfering with this enhancement of expression of IGFBPs and enhancing IGF-1 bioavailability, thus protecting cartilage from resorption.

JWH-133 and Win-55,212-2 appeared to attenuate IL-1-induced LTB₄ production in a concentration-dependent manner whilst HU-210 enhanced it. ACEA and N-AG also enhanced LTB₄ production in a concentration-dependent manner. Since N-AG also attenuated PGE₂ production, it may be speculated that inhibition of the COX pathway enhanced the activation of the 5-LOX pathway in this system. Other non-steroidal anti-inflammatory drugs (NSAIDs) also up-regulate the synthesis of LTB₄, supporting the shunt hypothesis from COX to 5-LOX (Martel-Pelletier *et al.*, 2004). Therefore these cannabinoids may be working in a similar way to the NSAIDs.

Inhibition of NO-sensitive GC by ODQ reduced LTB₄ production suggesting that LTB₄ production is dependent on cGMP production. Inhibition of COX-2 by NS-398 also blocked LTB₄ production in chondrocytes implying that COX pathway activation enhances LTB₄ production as well. Inhibition of NF-κB by PDTC, on the contrary, appeared to enhance LTB₄ production.

It is likely that LTB₄ contributes to the up-regulation of important catabolic factors involved in the pathophysiology of OA, such as MMP (Martel-Pelletier *et al.*, 2004). Therefore the effects of Win-55,212-2 and JWH-133 would be beneficial in treating OA.

The inhibition of prostaglandin synthesis has long been a basis for the treatment of OA, and other eicosanoids such as leukotrienes have also been implicated in the pathogenesis of osteoarthritis. Therefore selective inhibition

of prostaglandin pathways and/or inhibition of leukotriene activity may prove to be effective therapeutic strategies in osteoarthritis (Molloy and McCarthy, 2005).

Conclusion

Cannabinoids, N-AG, JWH-133 and Win-55,212-2 attenuated PGE₂ production induced by IL-1 significantly. Cannabinoids significantly reduce PGE₂ production in articular chondrocytes possibly through their ability to block COX-2 expression. Win-55,212-2 was shown to be a specific COX-2 inhibitor since it did not affect expression of COX-1. Cannabinoids appear to have a potential as therapeutic agents in OA partly through this ability to block the COX-2 pathway.

CHAPTER 6

EFFECTS OF CANNABINOIDS ON IL-1-INDUCED EXTRACELLULAR MATRIX DEGRADATION

6.1 Introduction

A central pathological feature of the arthritic diseases is the resorption of cartilage. Chondrocytes, maintain cartilage tissue homeostasis, controlling the turnover rate of cartilage extracellular matrix (Steinmeyer and Daufeldt, 1997). Active proteinases such as matrix metalloproteinases (MMPs) and aggrecanases degrade cartilage extracellular matrix (Cawston, 1998; Porter *et al.*, 2005). The proteinases are inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Steinmeyer and Daufeldt, 1997). 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 (Cawston, 1998; Steinmeyer and Daufeldt, 1997). Cartilage oligomeric matrix protein (COMP) and proteoglycan (mainly aggrecan) degradation occurs early in cartilage resorption process followed by collagen breakdown (Forslind *et al.*, 1992; Little *et al.*, 2002). Presence of COMP fragments in synovial fluid or serum is used as a marker for cartilage degradation (Neidhart *et al.*, 1997; Saxne and Heinegard, 1992). Effects of cannabinoids on IL-1-induced extracellular matrix resorption were investigated in bovine nasal cartilage explant cultures and expression of proteinases in conditioned media from bovine articular chondrocyte cultures.

6.2 Materials and methods

6.2.1 Cannabinoids: Endocannabinoid anandamide (Sigma-Aldrich), synthetic cannabinoids HU-210 (Tocris), Win-55,212-2 (active enantiomer) (Sigma-Aldrich) and Win-55,212-3 (inactive enantiomer) (Sigma-Aldrich) were studied along with solvent controls, ethanol and DMSO (0.1% final concentration) (Sigma, UK). Their effects were investigated on unstimulated or IL-1 α -stimulated cartilage breakdown.

6.2.2 Cartilage proteoglycan breakdown (sGAG assay): The cartilage explants were prepared and incubated overnight in complete medium, after which, the explants were unstimulated or stimulated to resorb with IL-1 α (500

U/ml \equiv 0.3 nM) in the presence of anandamide or Win-55,212-2 (5-50 μ M) for 4 days; HU-210 (0-15 μ M) for 21 days and Win-55,212-2 or Win-55,212-3 (0-10 μ M) for 28 days in DMEM without FBS. The rest of the assay was carried out as described in section 2.2.6.3.

6.2.3 Cartilage collagen breakdown (hydroxyproline assay): some of the conditioned culture media used in the sGAG assay above was set aside for this assay. Collagen degradation was determined as described under section 2.2.6.1.

6.2.4 COMP release assay: COMP release was determined using an animal COMP ELISA as described in section 2.2.6.2.

6.2.5 Statistical Analysis: Statistical significance was determined using Kruskal-Wallis non-parametric test followed by Dunn's post-hoc test and a $p < 0.05$ was considered statistically significant.

6.2.6 Zymography: This was carried out using manufacturer's pre-cast gels, buffers and instruction up to developing stage. Briefly, the zymogram gelatin gels which were 10% Tris-Glycine gels with 0.1% gelatin incorporated as substrate were used. Conditioned media from bovine articular chondrocyte cultured was obtained as described in section 2.2. The cells were stimulated with IL-1 in the presence or absence of Win-55,212-2 for 48 hrs. For the rest of the procedure refer to section 2.2.8.

6.3 Results

6.3.1 Preliminary studies on the effects of cannabinoids on IL-1-induced matrix degradation in bovine nasal cartilage (BNC).

The endocannabinoid anandamide and synthetic cannabinoid Win-55,212-2 were used in the preliminary studies on IL-1-induced cartilage degradation measured as the release of sulphated GAGs in 4-day cartilage explant cultures.

Explants were treated with IL-1 α (0.3nM) to induce matrix resorption. Effects of anandamide and Win-55,212-2 on IL-1-induced matrix release were studied. IL-1 α (0.3nM) significantly induced proteoglycan breakdown in bovine nasal cartilage (BNC) explants. Anandamide (10 and 50 μ M) (Fig. 6.1) and Win-55,212-2 (5, 10 and 50 μ M) (Fig. 6.2) significantly reduced IL-1 induced proteoglycan degradation in BNC cultured for 4 days. Their effects appeared to be concentration-dependent. Win-55,212-2 appeared to be the more potent than anandamide, although these were two different experiments.

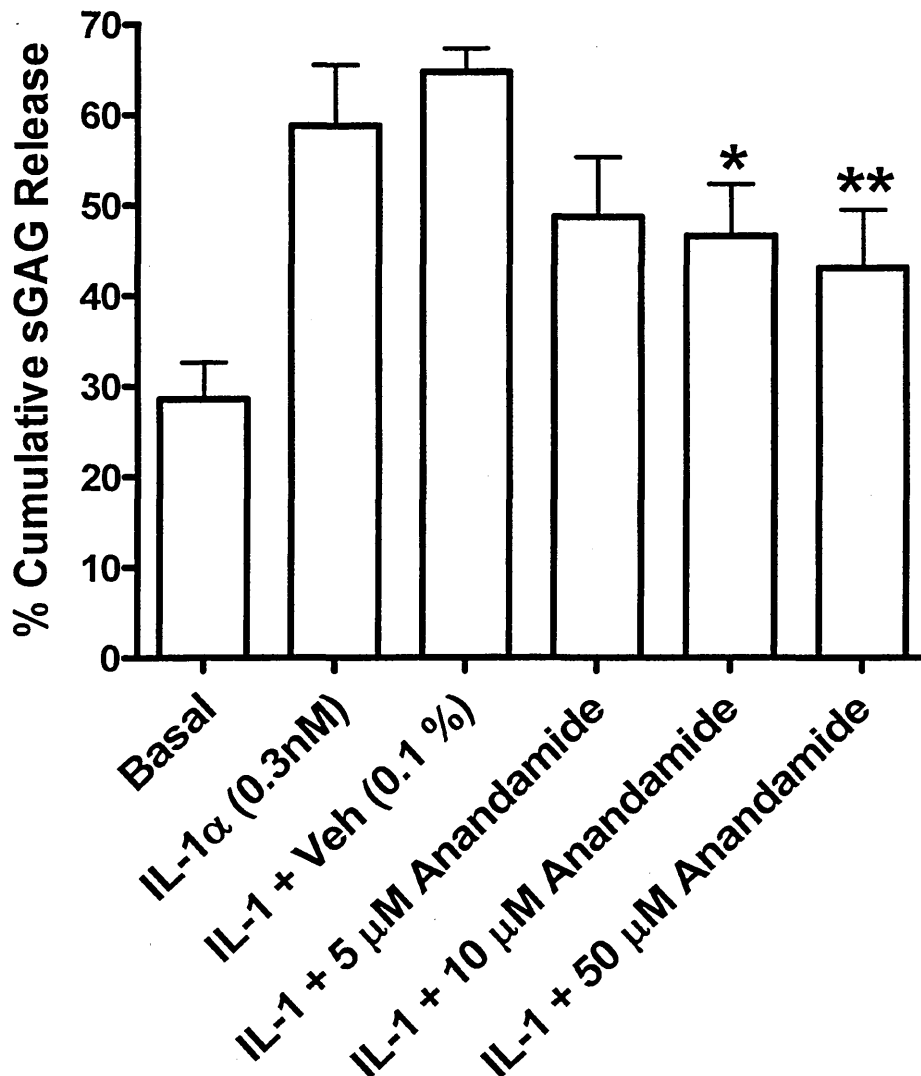


Figure 6.1: Effects of anandamide (5-50 μ M) on 4 day IL-1 α -induced sGAG release in bovine nasal cartilage explants. Results expressed as mean \pm s.e.m. (n=6). * p<0.05; **p<0.01 compared with vehicle control. Kruskal-Wallis

followed by Dunn's post-hoc test was used to determine statistical significance.

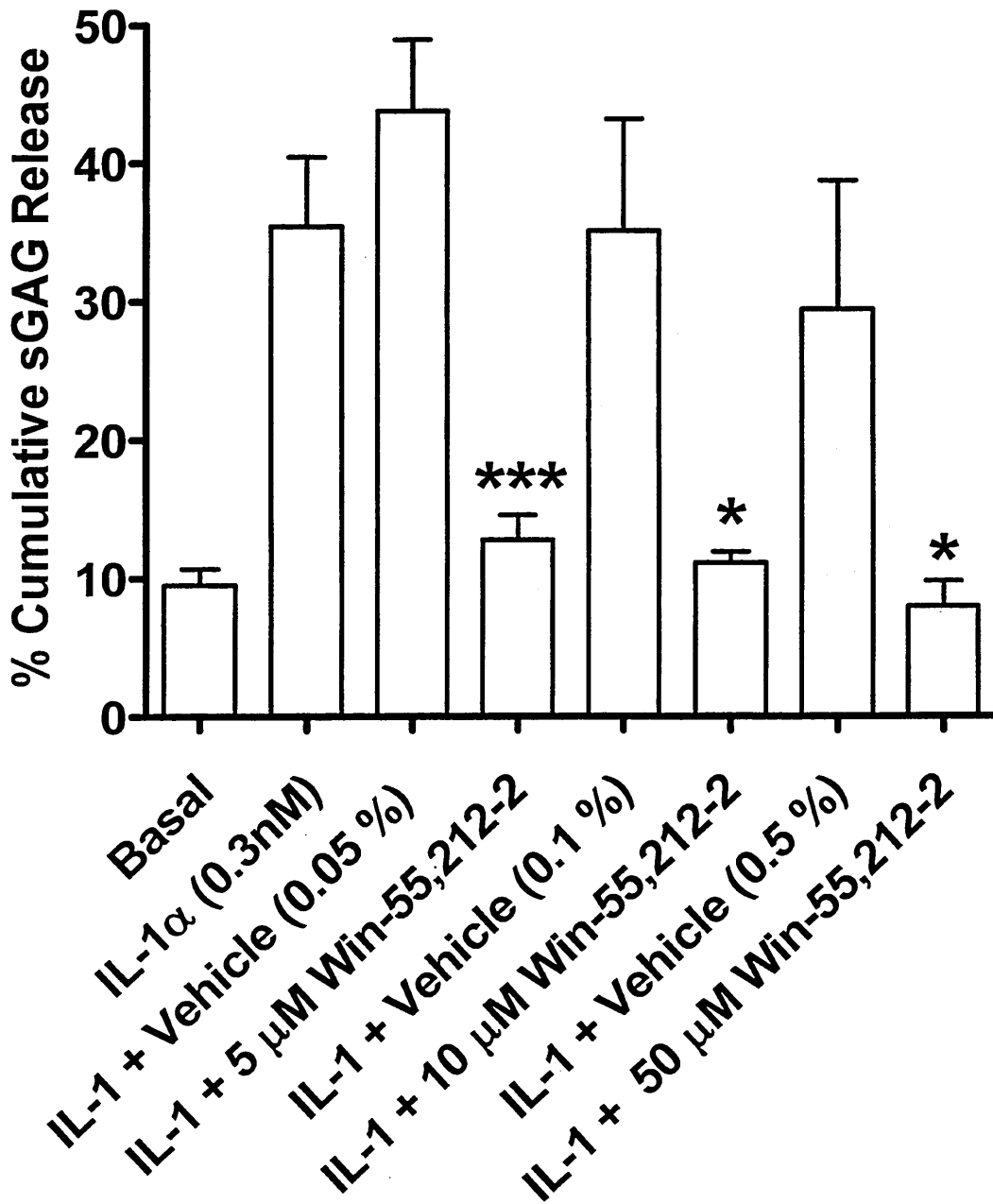


Figure 6.2: Effects of Win-55,212-2 (5-50 μ M) on 4 day IL-1 α -induced sGAG release in bovine nasal cartilage explants. Results expressed as mean \pm s.e.m. (n=6). * p<0.05; *** p<0.001 compared with vehicle control. Kruskal-Wallis followed by Dunn's post-hoc test was used to determine statistical significance.

Having shown that the cannabinoids were able to reduce matrix breakdown induced by IL-1, longer duration cultures of the BNCs were carried out with the intention of studying the effects of cannabinoids on proteoglycan and collagen degradation to the point where the cartilage explants in the control wells were completely resorbed through IL-1 induced pathways.

6.3.2 Effect of HU-210 and Win-55,212-2 on IL-1 α -induced proteoglycan degradation in bovine nasal cartilage explants

Bovine cartilage explants were incubated for up to 21 or 28 days stimulated with IL-1 in the presence and absence of HU-210, the active enantiomer Win-55,212-2 and the inactive enantiomer Win-55,212-3. The conditioned media from these cultures were used to determine the release of proteoglycan measured as sGAGs. The effects of the enantiomers were compared to determine whether cannabinoids may be blocking proteoglycan breakdown via any receptor(s).

HU-210 (5-15 μ M) reduced sGAG release at days 7-14 with approximately 20% difference compared with the vehicle controls. HU-210 at 15 μ M significantly ($p < 0.05$) reduced sGAG release at day 7 (Fig. 6.3). By day 7, about 80 % of the proteoglycan had been lost through IL-1 induced mechanisms.

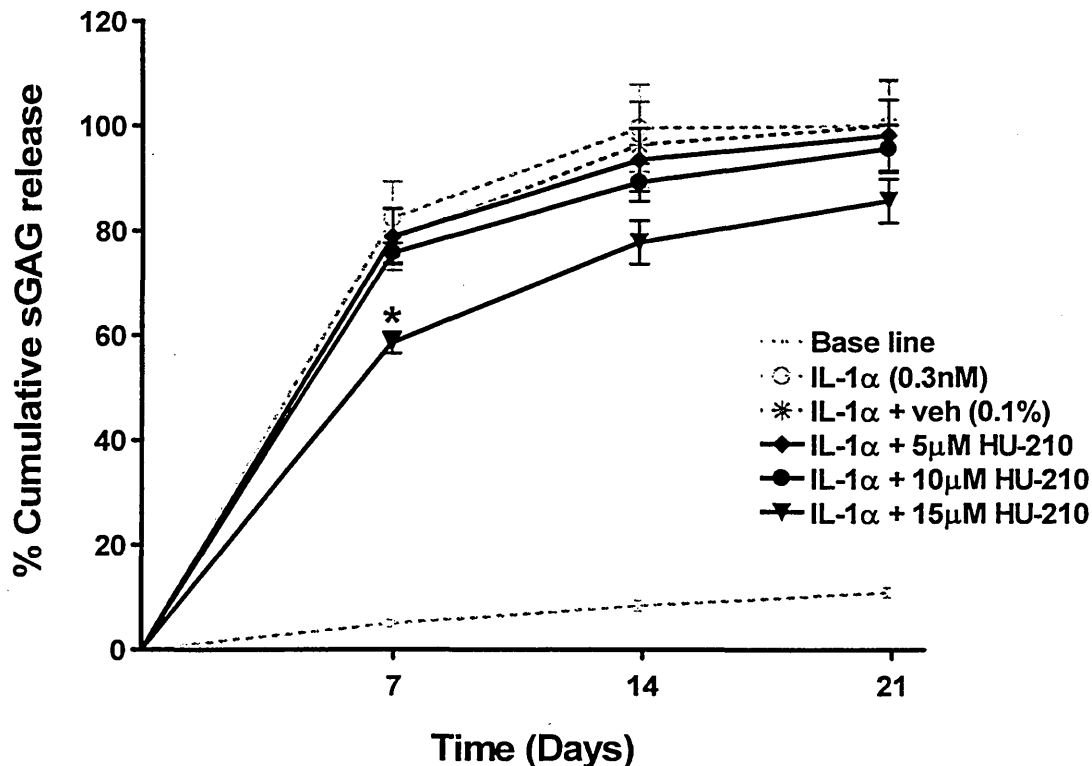


Figure 6.3: The effect of HU-210 on IL-1-induced proteoglycan degradation in bovine nasal cartilage explants. Results expressed as mean \pm s.e.m. (n=6). * $p < 0.05$ compared with vehicle control.

The inactive enantiomer, Win-55,212-3, did not show any effect on sGAG release when compared to the vehicle control whilst Win-55,212-2 at 5 and 10 μ M significantly ($p < 0.001$) reduced sGAG release compared with the vehicle control and the reduction was to basal levels (Fig. 6.4). Actually, Win-55,212-2 kept sGAG release to basal levels through out the culture period. By the end of day 7, approximately 80 % of the proteoglycan had been lost in the IL-1 treatment; over 40 % had been lost in the vehicle and Win-55,212-3 treatments.

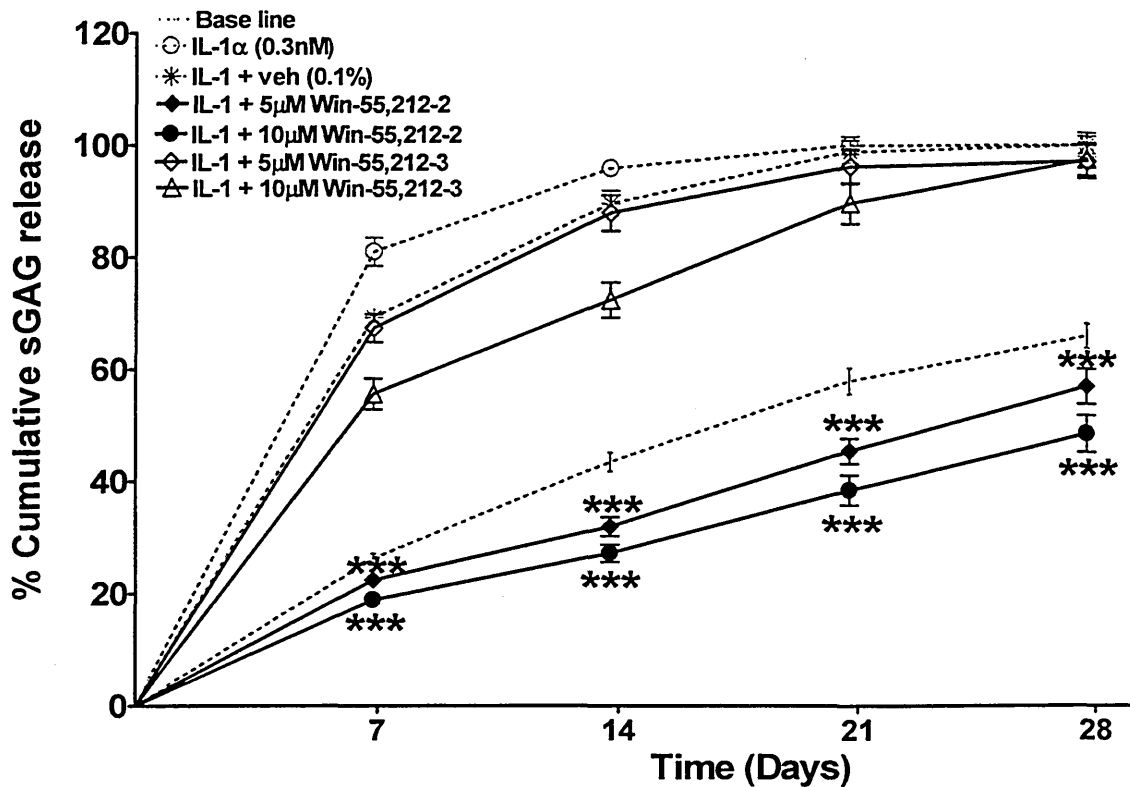


Figure 6.4: The effect of Win-55,212-2 compared with its inactive enantiomer, Win-55,212-3 on IL-1 α -induced proteoglycan degradation in bovine nasal cartilage explant cultures. Results expressed as mean \pm s.e.m. (n=6). *** p<0.001 compared with vehicle control. Kruskal-Wallis followed by Dunn's post test was used to determine statistical significance.

6.3.3 Effects of cannabinoids on IL-1 α -induced collagen degradation in bovine nasal cartilage explants

The conditioned media from bovine cartilage explant stimulated with IL-1 in the presence or absence of HU-210; and the enantiomers, Win-55,212-2 (active) and Win-55,212-3 (inactive) were used to determine the release of collagen measured as HPro. The effects of the enantiomers were compared to determine whether cannabinoids may be blocking collagen breakdown via any receptor(s).

At 5 μ M, HU-210 showed no effect at day 7 but later slightly reduced hydroxyproline release by up to 20% compared with the vehicle control at

days 14 and 21. At 10 and 15 μM , however, HU-210 significantly reduced hydroxyproline release at day 7 ($p < 0.01$ and $p < 0.05$ respectively) and at days 14 and 21 ($p < 0.001$ and $p < 0.01$ respectively) (Fig. 6.5). Only about 20 % of the collagen in the explants treated with IL-1 had been lost by the end of day 7. By day 21, the explants in the IL-1 and vehicle controls were completely resorbed.

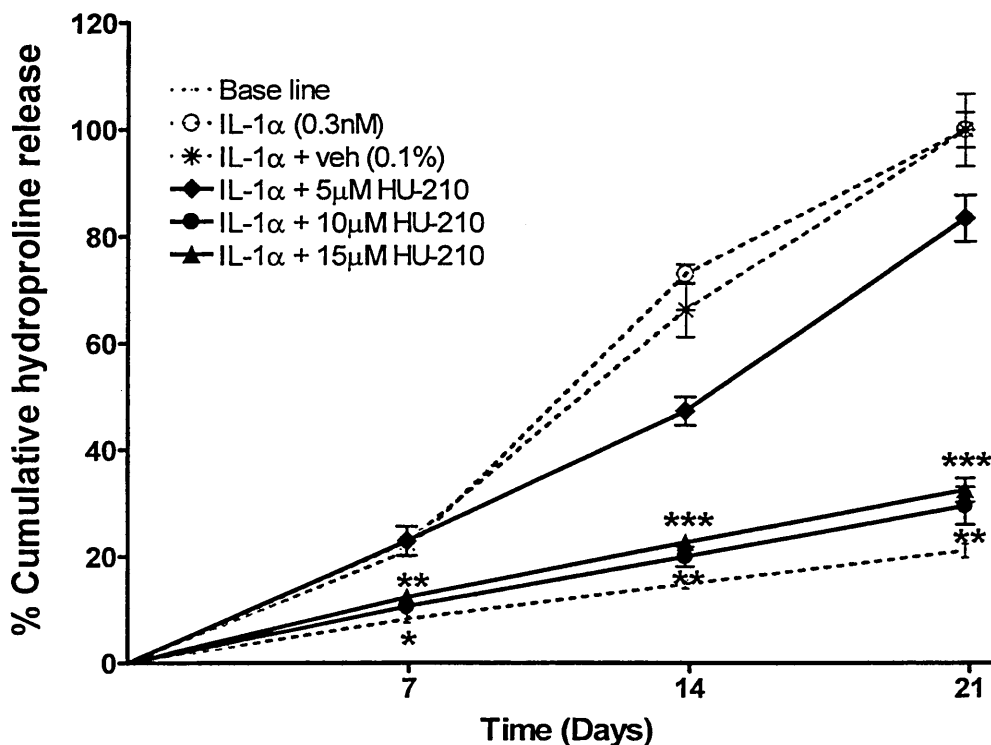


Figure 6.5: The effect of HU-210 on IL-1-induced collagen breakdown in bovine nasal cartilage explants. Results expressed as mean \pm s.e.m. ($n=6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with vehicle control. Kruskal-Wallis followed by Dunn's test was used to determine statistical significance.

The inactive enantiomer Win-55,212-3 did show some inhibitory effects on hydroxyproline release although the effects were not significantly different compared with those of vehicle control. However at 5-10 μM , Win-55,212-2 significantly ($p < 0.001$) reduced hydroxyproline release compared to the vehicle control (Fig. 6.6). Less than 20 % of the explants' collagen had been lost through IL-1-induced mechanisms by the end of day 7. By day 28, the

explants were completely resorbed in the IL-1 and vehicle control (DMSO) treatments.

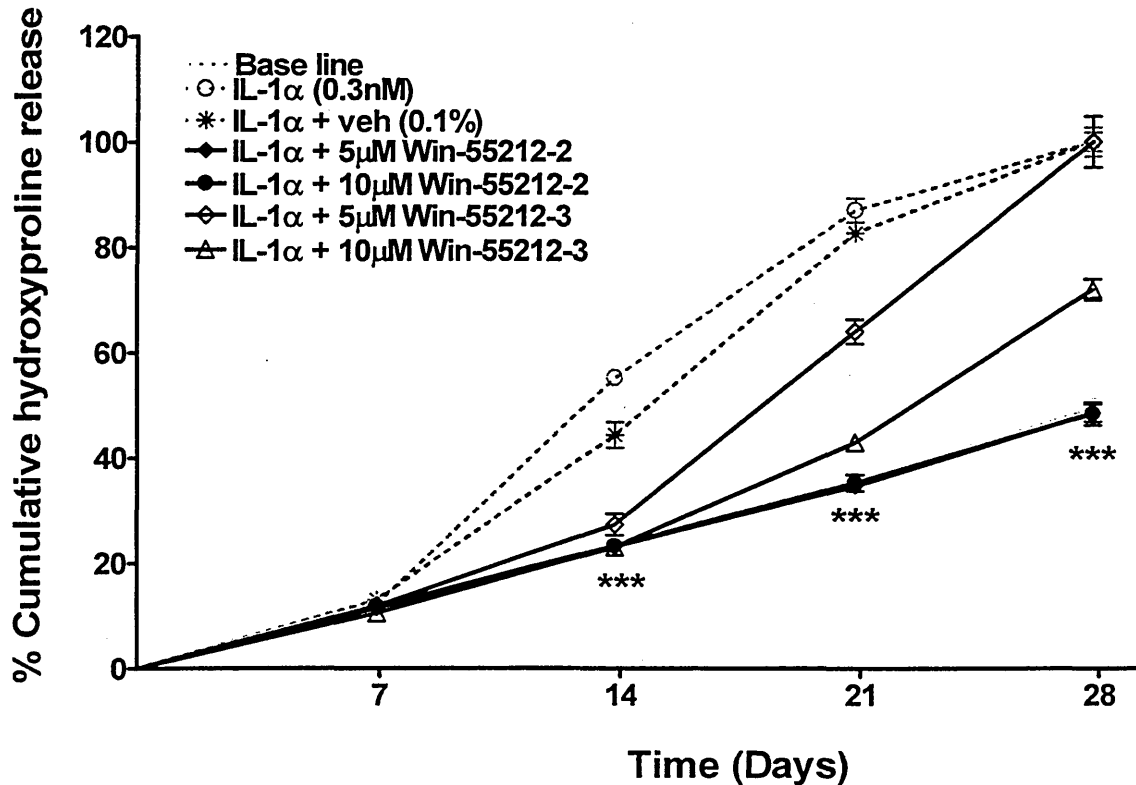


Figure 6.6: The effect of Win-55,212-2 compared with its inactive enantiomer, Win-55,212-3 on IL-1 α -induced collagen degradation in bovine nasal cartilage explant cultures. Results expressed as mean \pm s.e.m. (n=6). *** p<0.001 compared with vehicle control. Kruskal-Wallis followed by Dunn's test was used to determine statistical significance.

6.3.4 Effect of cannabinoids on IL-1-induced COMP release in articular cartilage

The release of COMP was measured in conditioned media from BNC explants that were stimulated with IL-1 and treated with Win-55,212-2 for 28 days. The effect of Win-55,212-2 was studied on the release of COMP.

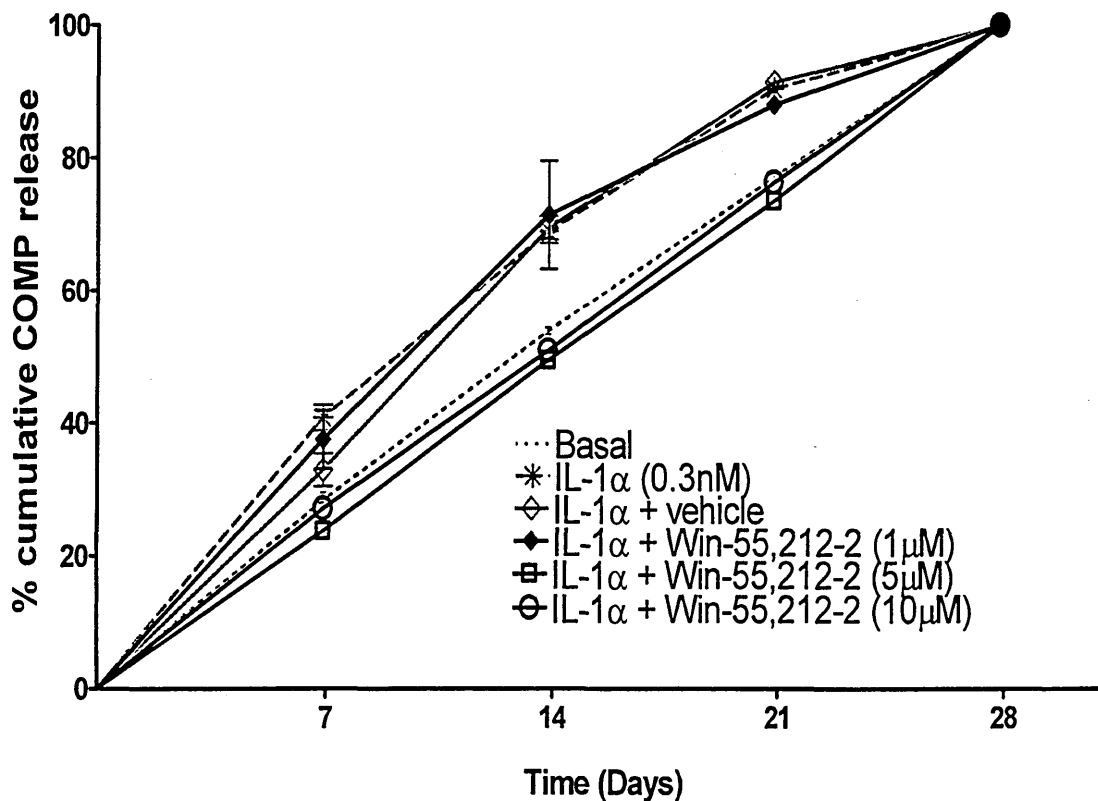


Figure 6.7: The effect of Win-55,212-2 (1, 5 and 10 μ M) on IL-1-induced COMP degradation in bovine nasal cartilage explants. The results are expressed as mean \pm s.e.m. (n=6).

Win-55,212-2 appeared to consistently inhibit the release of COMP throughout the culture period. Win-55,212-2 kept the release of COMP to basal levels (Fig. 6.7). This implies that Win-55,212-2 blocked IL-1-induced matrix breakdown completely. However, the results show that basal release of COMP was almost in a linear fashion, ending with 100% release at day 28, just as the rest of treatments in which COMP release was induced by IL-1 α . This is an anomaly, basal levels of release of COMP should have been much lower than the levels observed. The results observed could be attributed to loss of remaining COMP in the explants digested with papain solution, since no COMP residues were detected in the digests. Papain digestion of COMP may be responsible for the loss of the protein. Because the calculations were done as a percent of total COMP, loss of COMP in the digests distorted the results.

6.3.5 The effect of cannabinoid, Win-55,212-2 on proteases expression

The effects of Win-55,212-2 on expression of proteases in condition media from articular chondrocyte culture were investigated using 10% zymogram gelatin gels. Presence of proteases is readily visualised as clear bands in the lanes against the blue background. Clear bands represent the bands where proteases digested the gelatin. This study would enable identification of gelatinases such as matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) and other proteinases that can digest gelatin.



Figure 6.8: 10% Gelatin zymogram gels were used to detect proteases that can utilise gelatine as substrate in bovine articular chondrocyte conditioned media. The cells were unstimulated or stimulated with IL-1 and incubated for 48 h in the presence or absence of Win-55,212-2 (1-10 pM) or its vehicle. Lane 1 & 2: Markers; Lane 3: Blank; Lane 4: Basal; Lane 5: IL-1a (0.06nM); Lane 6: Vehicle control; Lane 7: IL-1+ Win-55,212-2 (10pM); Lane 8; IL-1+ Win-55,212-2 (5pM); Lane 9: IL-1+ Win-55,212-2 (1pM)

The conditioned media contained a number of proteases which may include the well-known gelatinases, MMP-2 and MMP-9 apparently in their inactive and/or active forms (Fig. 6.8). The molecular weights for the active MMP-2, pro-MMP-2, active MMP-9 and pro-MMP-9 are about 65, 72, 92 and 100 kDa, respectively. MMP-9, active form does not appear to be expressed in this

system, however there appears to be a faint band at above 97 kDa, which could be the pro-MMP-9. Win-55,212-2 appears to reduce expression of this form of gelatinase in a concentration-dependent manner (Fig 6.8, lanes 7, 8 & 9). There is a faint band above and a clear pronounced band around 64 kDa. The one above 64 kDa may be the Pro-MMP-2 which appeared not to be affected by presence of IL-1 or Win-55,212-2 and was not as highly expressed as the one around 64 kDa which may be the active form of MMP-2 (Fig. 6.8). Expression of the “active MMP-2” appears to be slightly enhanced by presence of IL-1, but was attenuated by Win-55,212-2 at 5 and 10 μ M. Other protease activities were around 45 kDa and below (Fig. 6.8), it is possible that these may represent MMP-1/MMP-3 as suggested elsewhere (Collen *et al.*, 2003). The molecular weights for pro-MMP-1, MMP-1, pro-MMP-3 and MMP-3 are: 51 or 53 kDa, 41 or 44 kDa, 43 or 45 kDa, and 57 kDa, respectively (Fuchs *et al.*, 2004). These may vary depending on glycosylation state. The expression of these proteases was not affected by IL-1 stimulation but was abrogated by Win-55,212-2 in what appeared to be a concentration-dependent manner, with significant effects from 5 μ M. With zymography, it is difficult to identify the proteases with accuracy and certainty especially for those proteases with nearly similar molecular weights (Fuchs *et al.*, 2004).

Conditioned media from explant cultures were also tested but there was a problem (results not shown). The presence of released matrix components such as glycosaminoglycans appeared to impede migration of the enzymes and caused smearing of gels. No clear bands were visualised in conditioned media from days 7-14 cultures, but slightly clearer bands were seen in conditioned media from days 21-28 (data not shown). The bands became clear in days 21-28 culture media, particularly in lanes for IL-1 α (0.3nM), vehicle control as well as 1 μ M Win-55,212-2, showing proteases expression. The lanes for basal and 5 μ M Win-55,212-2 showed little, if any expression of the proteases. This suggested that by day 21, the conditioned media in the lanes that showed clear protease activity bands had less presence of the degraded components of the matrix. This further indicated that there was accelerated and enhanced matrix resorption in those treatments during the 14

days of culture. The smearing of gels that was seen was more associated with the conditioned media from the IL-1, vehicle control and 1 μ M Win-55,212-2 treatments.

6.4 Discussion

The results show that synthetic cannabinoids have the potential to protect against IL-1 α -induced cartilage breakdown as evidenced by inhibition of cartilage matrix degradation in bovine nasal cartilage explants. The synthetic cannabinoids significantly inhibited proteoglycan degradation early in the process of cartilage resorption and potently inhibited collagen breakdown later in the process (see Figures 6.1-6.6). HU-210 and Win-55,212-2 significantly inhibited IL-1-induced proteoglycan and collagen breakdown in bovine cartilage explants. Win-55,212-2 was also capable of blocking IL-1 induced COMP release in this system. Win-55,212-2 was able to reduce matrix degradation to about basal levels at the concentrations tested, thus showing ability to abrogate completely the degradative effects of IL-1 in cartilage. This effect of Win-55,212-2 may be partly due to its ability to reduce expression of proteinases. Generally, the proteinases expressed in the conditioned media were constitutively expressed. However these results suggest that Win-55,212-2 blocked cartilage breakdown by attenuating expression of proteases. The MMPs are a unique family of enzymes that are responsible for catabolism of extracellular matrix, working in concert; these proteases can degrade all components of the extracellular matrix (Ellis *et al.*, 1994; Flannery *et al.*, 2000).

In the system used, proteoglycan release appeared to occur earlier in the cartilage resorption process; IL-1 induced the release of approximately 80 % of the proteoglycan in the explants by the end of day 7. For COMP it was approximately 40 % and for collagen only about 20 % by the end of the first week of culture.

The effects of the cannabinoids in these studies appeared to be generally concentration-dependent. The stereo-specificity exhibited by the effects Win-

55,212-2, the active enantiomer at cannabinoid receptors, on these cartilage resorption assays suggests that Win-55,212-2 was acting via CB₁ and/or CB₂ cannabinoid receptor(s).

It is not known for certain, how the cartilage resorbed in the presence of IL-1 in the assays studied. However, it was shown in chapters 4 and 5 that IL-1 significantly stimulated production of the inflammatory mediators, NO and PGE₂ respectively. The mediators appear to be partly responsible for the effects of IL-1 observed. They have been reported to induce apoptosis in chondrocytes (Miwa *et al.*, 2000 and Notoya *et al.*, 2000). Blanco *et al.* (1998) carried out studies to determine the kind of cell death which occurred in cartilage from patients with OA. These studies revealed that chondrocytes in OA cartilage undergo apoptosis. This mechanism of cell death plays an important role in the pathogenesis of OA (Blanco *et al.*, 1998). Chondrocyte loss has profound effects on matrix composition; on the other hand, matrix composition has an equally profound effect on chondrocytes viability (Lo and Kim, 2004). Chondrocyte apoptosis has been described in studies of the pathogenesis of various arthritides and collagen framework degradation has been shown to induce apoptosis of chondrocytes in human cartilage (Kim *et al.*, 2001). Therefore, collagen degradation appears to be a physiologically relevant pro-apoptotic signal and, quite likely, a key mediator of chondrocyte apoptosis in diseases such as osteoarthritis (Lo and Kim, 2004). Therefore the possibility of induction of chondrocyte apoptotic death by matrix breakdown can not be ruled out in this present study. It is possible that IL-1-induced activation of proteases that started the degradation of the matrix. This in turn, stimulated chondrocytes death and, this in turn led to compromised extracellular matrix integrity, triggering a vicious cycle. Whichever starts, remains to be elucidated.

Conclusion

Cannabinoids blocked proteoglycan, COMP and collagen degradation induced by IL-1 α in bovine nasal cartilage explants. Although IL-1 α completely resorbed cartilage explants in 3 – 4 weeks time, cannabinoids

protected the cartilage explants from the IL-1-induced breakdown. This could be partly through inhibition of proteinase expression. Finally cannabinoids could be acting through some receptor(s).

CHAPTER 7

THE MODE OF ACTION OF CANNABINOIDS IN CHONDROCYTES

7.1 Introduction

The p38 MAPK signalling transduction pathway plays an essential role in regulating many cellular processes including inflammation, cell differentiation, cell growth and death. Activation of p38 often through extracellular stimuli such as bacterial pathogens and cytokines, mediates signal transduction into the nucleus to turn on the responsive genes (Ono and Han, 2000).

MAPK p38 is strongly activated by pro-inflammatory stimuli, and has been studied largely in the context of inflammation (Clark *et al.*, 2003). Activation of p38 MAPK has been implicated in the catabolic and anti-anabolic actions of IL-1 (Studer *et al.*, 2004). Activation of p38 plays an important role in the induction and expression of inflammatory mediators such as COX-2 and iNOS (Ono and Han, 2000). Inhibition of this pathway may be a versatile anti-inflammatory strategy and this accounts for the intense interest of the pharmaceutical industry in the development of p38 MAPK inhibitors (Clark *et al.*, 2003).

Another important way to block induction and expression of mediators of inflammation and tissue resorption such as NO, PGE₂ and pro-inflammatory cytokines, would be to target the transcription factors responsible for induction of the genes of these mediators in the nucleus. NF-κB is one such transcription factor that could be targeted. This would allow the specific control of the overexpression of a number of genes that occur in many arthritic conditions like OA (Pelletier, 2004). The NF-κB/Rel family of transcription factors consists of five known proteins: p65, p50, p52, c-Rel and RelB. All NF-κB proteins possess a conserved amino-terminal domain that contains the DNA-binding and dimer-forming domains, as well as the nuclear localization signal (NLS). Active NF-κB molecules exist as dimers, mostly in the form of p65/RelA and p50 (Schwartz *et al.*, 1999). In unstimulated cells, NF-κB is sequestered in the cytoplasm by the inhibitor of kappaB (IκB) proteins, thus it exists as a heterotrimer of p50, p65 and IκB. IκB prevents nuclear translocation and DNA binding of NF-κB by masking its NLS (Shishodia and Aggarwal, 2004). In response to proinflammatory stimuli, IκB is phosphorylated (Clancy *et al.*, 1998). The phosphorylation leads to

ubiquitination and degradation of I κ B, thus unmasking the NLS and allowing NF- κ B to translocate to the nucleus where the NF- κ B dimers bind to the promoter region of targeted genes and regulates their transcription (Clancy *et al.*, 1998; Schwartz *et al.*, 1999). The composition of the dimer and the cell type in which it is functioning are thought to contribute to the transcriptional specificity of NF- κ B (Schwartz *et al.*, 1999).

Cannabinoids appear to be acting through receptor(s) in the systems used in this study, as evidenced by effects that are apparently concentration-dependent and the stereo-specificity of effects of Win-55,212-2. Therefore expression of cannabinoid receptors in chondrocytes was studied. The effects of Win-55,212-2 on IL-1 induced activation of the p38 MAPK pathway as well as activation of the NF- κ B were studied in bovine articular chondrocytes.

7.2 Materials and methods

7.2.1 Materials: MK-886, inhibitor of 5-lipoxygenase (5-LOX); NS 398, inhibitor of cyclooxygenase 2 (COX-2); ODOJ, inhibitor of NO-sensitive guanylyl cyclase; and pyrrolidine dithiocarbamate ammonium (PDTTC), inhibitor of NF- κ B; PGE₂ and LTB₄ ELISA kits (Alexis Biochemical); FACE[®] MAPK p38 kit; rabbit polyclonal anti-cannabinoid receptors - CB₁ (H-150) and CB₂ (H-60) (Santa Cruz); rabbit polyclonal anti-NF- κ B p65 (1:100) (SantaCruz) and fluorescein isothiocyanate (FITC)-conjugated secondary goat anti-rabbit IgG (Santa Cruz) diluted (1:100) were used in this study.

7.2.2 Bovine chondrocyte culture: Chondrocytes stimulated with IL-1 in the presence or absence of Win-55,212-2 (0-10 μ M) were cultured as describe in section 2.2.1.

7.2.3 FACE[™] p38 MAPK: This Fast-activated cell-based ELISA for p38 MAPK was carried out as described under section 2.2.10

7.2.4 Immunofluorescence: Chondrocytes were plated on 8-well chamber slides and incubated for 5 days in complete medium (for cannabinoid receptor

immunofluorescence) or incubated for a further 48 hours stimulated with IL-1 α (0.06nM) in the presence of Win-55,212-2. The rest of the procedure was carried out as described in section 2.2.9. Rabbit polyclonal anti-cannabinoid receptors - CB₁ (H-150) and CB₂ (H-60) (Santa Cruz) and rabbit polyclonal anti-NF- κ B p65 (1:100) (SantaCruz) were used. Fluorescein isothiocyanate (FITC)-conjugated secondary goat anti-rabbit IgG (Santa Cruz) diluted (1:100) in PBS with NGS was used to label the cells. Normal goat serum was used as a negative control. Cells were mounted in *SlowFade* anti-fade fluorescent mounting medium (Molecular Probes).

7.2.5 Immunoblotting: whole cell lysates were obtained from chondrocytes incubated with or without IL-1 α stimulation in the presence or absence of cannabinoid, Win-55,212-2 (1 or 5 μ M) for 48 hrs. The procedure was carried out as described in section 2.2.7. Briefly, after blocking, the membranes were incubated in primary antibodies - rabbit polyclonal anti-cannabinoid receptor-1 and -2 (CB₁ and CB₂) (Sigma-Aldrich) for 1 hr. After washing the membranes were incubated in alkaline phosphatase-conjugated goat anti-rabbit secondary antibody for 30 min followed by visualized of purple protein bands with chromogenic substrate [5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitro blue tetrazolium (NBT)] for at least 1hr. This was followed by imaging.

7.2.6 Statistical Analysis: Statistical significance was determined using one-way ANOVA followed by Boniferroni's post test and a $p < 0.05$ was considered statistically significant.

7.3 Results

7.3.1 Effect of Win-55,212-2 on IL-1-induced activation of p38 Mitogen-Activated Protein Kinase (MAPK) in bovine articular chondrocytes

Chondrocytes were cultures in 96 well-plates until at or near to confluence. The chondrocytes were stimulated with IL-1 and incubated with Win-55,212-2 at 1-10 μ M for 15 minutes. Activation of p38 MAPK in IL-1-stimulated

chondrocytes was studied and the effects of cannabinoid Win-55,212-2 were determined. Expression of total p38 MAPK was also determined and effects of Win-55,212-2 on this were studied as well.

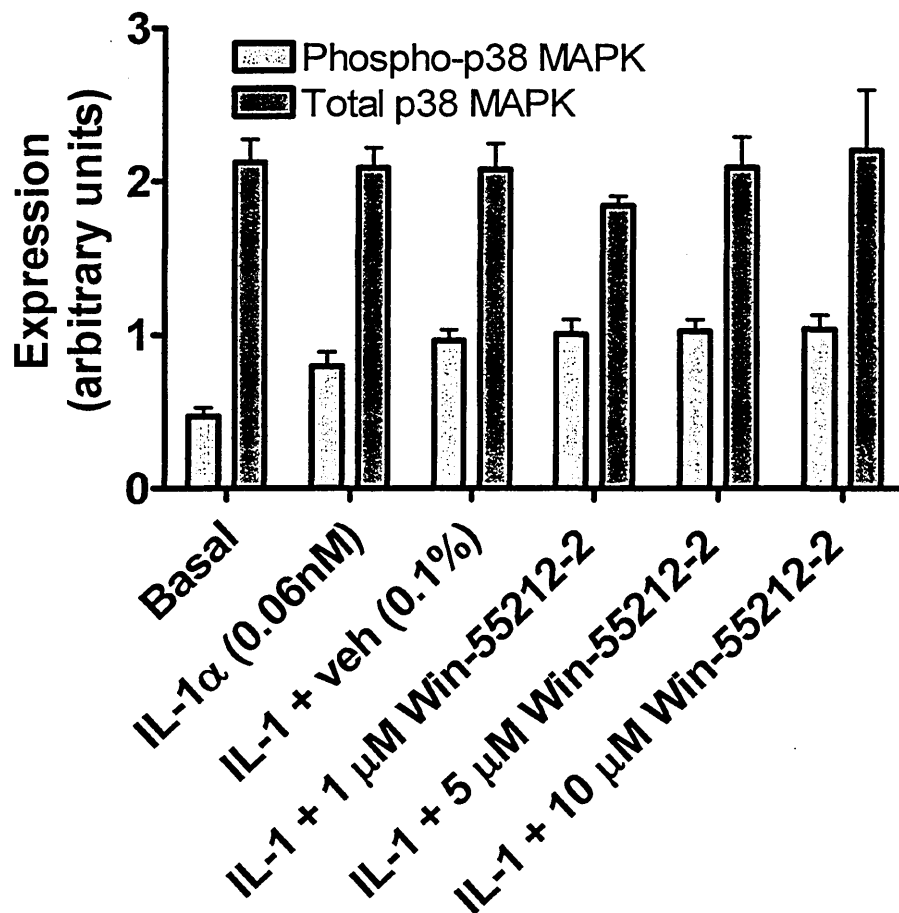


Figure 7.1: The effect of Win-55,212-2 (1, 5 and 10 μ M) on IL-1-induced activation of p38 MAPK in bovine articular chondrocytes incubated for 15 min. Results expressed as mean \pm s.e.m. (n=6).

IL-1 α stimulated phosphorylation of p38 MAPK in these chondrocytes (Fig. 7.1). Win-55,212-2 at 1-10 μ M did not show any significant effect on IL-1-stimulated phosphorylation of p38 MAPK (Fig. 7.1). Total p38 MAPK was not significantly affected by any of the treatments in these cells at 15 min following stimulation by IL-1 (Fig. 7.1).

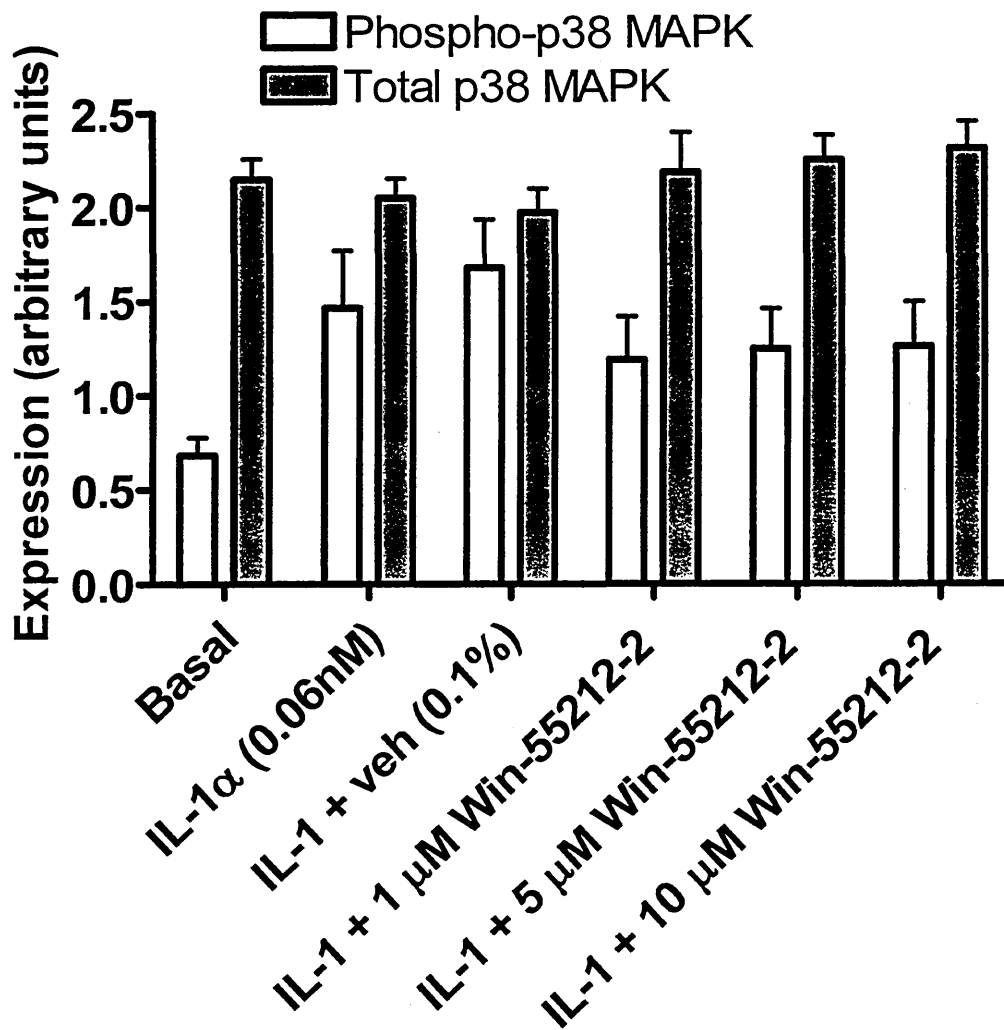


Figure 7.2: The effect of Win-55,212-2 (1, 5 and 10 μ M) on IL-1-induced activation of p38 MAPK in bovine articular chondrocytes incubated for 30 min. Results expressed as mean \pm s.e.m. (n=6).

IL-1 α induced the phosphorylation of p38 MAPK in these cells stimulated for 30 min (Fig. 7.2). The vehicle appeared to slightly enhance the phosphorylation; however, Win-55,212-2 at the concentrations tested equally inhibited the IL-1-induced phosphorylation of p38 MAPK (Fig. 7.2). The total p38 MAPK was not affected by the IL-1 or Win-55,212-2 but appeared to be slightly inhibited by the vehicle (Fig. 7.2).

7.3.2 Win-55,212-2 and NF- κ B activation in bovine articular chondrocytes

Bovine articular chondrocytes were plated onto chamber slides and were used in the study of effects of Win-55,212-2 on IL-1 α -induced NF- κ B activation using indirect immunofluorescence. A primary antibody against the p65 subunit of the NF- κ B dimers was used.

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Figure 7.3: Immunofluorescence analysis of NF- κ B activation in bovine articular chondrocytes cultured for 7 days. The cells were unstimulated (B) or stimulated with IL-1 α (0.06 nM) in the presence of: 0.1 % vehicle - DMSO (C); 5 pM Win-55,212-2 (D). (A) Negative control (NGS used instead of primary antibody). Primary antibody against NF- κ B p65 was diluted 1:100 and FITC-conjugated secondary antibody was used to visualise NF- κ B activation.

Under basal conditions, NF- κ B was expressed mainly in the cytoplasm (Fig. 7.3 B). Stimulation with IL-1 α activated the NF- κ B in chondrocytes as evidenced by the translocation of the transcription factor into the nuclei shown

by strong staining of the nuclei, little staining in the cytoplasm (Fig. 7.3 C). Win-55,212-2 (5 pM) attenuated the IL-1-induced activation of NF- κ B in bovine articular chondrocytes (Fig. 7.3 D) as shown by reduced nuclei staining and general expression.

7.3.3 Cannabinoid receptor expression in bovine articular chondrocytes

The bovine articular chondrocytes were studied to determine expression of cannabinoid receptors using immunofluorescence and immunoblot analyses.

Figure 7.4: Immunofluorescence analysis of CB1 and CB2 cannabinoid receptors in bovine articular chondrocytes cultured for 7 days. A & C: negative controls (NGS used instead of primary antibody); B: CB1 receptor expression; D: CB2 receptor expression. Primary antibodies against the receptors were diluted 1:100. FITC-conjugated secondary antibody was used to visualise the receptor's expression.

In this immunofluorescence analysis, the chondrocytes showed punctate expression of the cannabinoid receptors, CB₁ and CB₂ (Fig. 7.4). The expression of these receptors appeared to be constitutive. The expression of the two cannabinoid receptors CB₁ and CB₂ by chondrocytes was confirmed also by immunoblot analyses (Fig. 7.5 and 7.6). These are unprecedented findings.

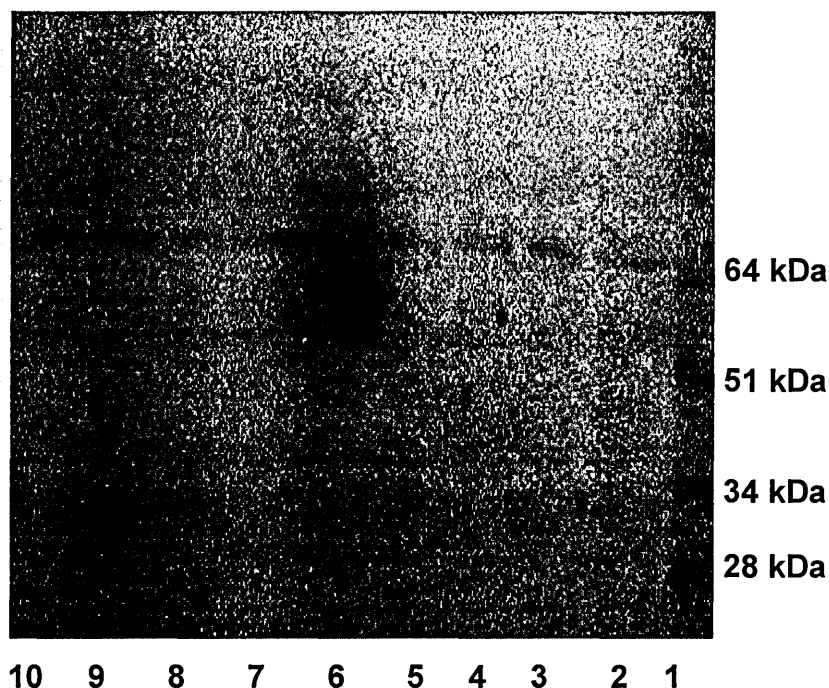


Figure 7.5: Immunoblot assay performed using anti-cannabinoid receptor-1 (CB₁) antibody from rabbit (diluted 1:250 as outlined by the manufacturer) in bovine chondrocyte lysate. Lanes 1: Marker; Lane 2: Basal; Lane 3: IL-1 α (0.06nM); Lanes 4: Vehicle control; Lanes 5 & 6: IL-1 + Win-55,212-2 (1 μ M); Lanes 7 & 8: IL-1 +Win-55,212-2 (5 μ M); Lane9 & 10: IL-1 +Win-55,212-2 (5 μ M)

The antibody against CB₁ cannabinoid receptor, when used in immunoblot recognises a major band of 60 kDa, according to information from the manufacturer. In this study, the antibody recognised a major band around 64 kDa. The bands observed were faint though, suggesting either low affinity for the antibody in this species or low expression of receptors.

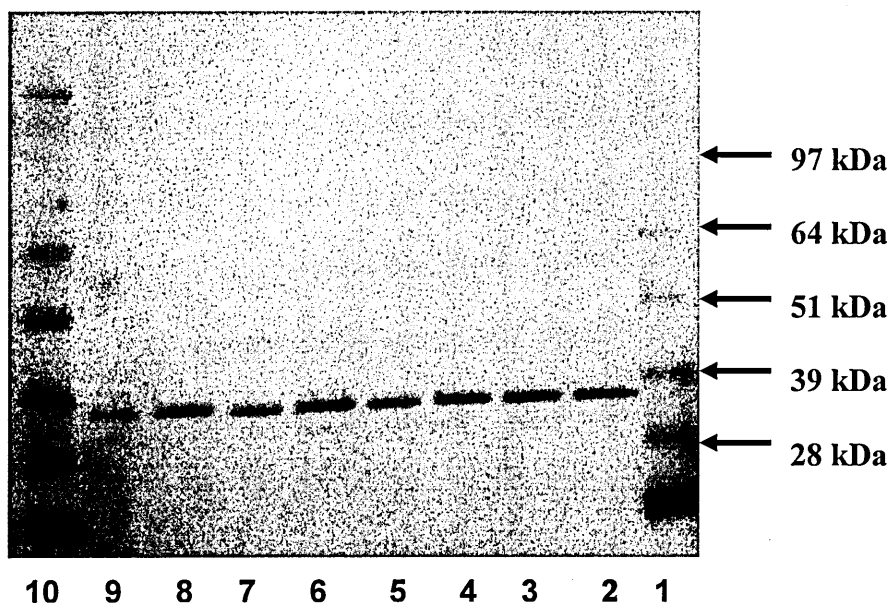


Figure 7.6: Immunoblot assay performed using anti-cannabinoid receptor-2 (CB₂) antibody from rabbit (diluted 1:500) in bovine chondrocyte lysate. Lanes 1: Marker; Lane 2: Basal; Lane 3: IL-1 α (0.06nM); Lanes 4 (30 μ l) & 5 (15 μ l): Vehicle control; Lanes 6 (30 μ l) & 7 (15 μ l): IL-1 + Win-55,212-2 (1 μ M); Lanes 8 (30 μ l) & 9 (15 μ l): IL-1 +Win-55,212-2 (5 μ M); Lane 10: Marker

The immunoblot for CB₂ cannabinoid receptors showed a major band at 30 kDa and a minor (faint) band at 97 kDa.

7.4 Discussion

The p38 MAP kinase inhibitors have been shown to be efficacious in several disease models, including inflammation, arthritis and other joint diseases. In all cases, p38 activation in key cell types correlated with disease initiation and progression. Treatment with p38 MAP kinase inhibitors attenuated both p38 activation and disease severity (Lee *et al.*, 2000). A number of compounds that inhibit p38 MAPK have been developed and their potential as therapeutic agents in inflammation, arthritis, septic shock, and myocardial injury is currently being explored (Studer *et al.*, 2004). Compounds that potently inhibit p38 MAPK have been found to be capable of inhibiting IL-1 induction of iNOS in bovine chondrocytes and thus blocking NO synthesis (Studer *et al.*, 2004).

This effect may also protect cartilage from the damaging actions of NO. It is possible that inhibition of activation of p38 MAPK by Win-55,212-2 consequently blocked the induction and expression of COX-2 and iNOS, resulting in inhibition of PGE₂ and NO production respectively. Therefore this ability of Win-55,212-2 to reduce p38 MAPK activation suggests that it has the potential as an effective therapeutic agent for arthritis.

In chondrocytes, NO is a key regulator of the signaling pathways downstream of IL-1, involving NF-kappa B (NF-κB) activation and leading to the expression of genes that are involved in the pathophysiology of arthritic diseases (Mendes *et al.*, 2002). NO is not per se involved in the immediate cytokine-stimulated activation of NF-κB in chondrocytes, but rather sustains nuclear translocation and this persistent activation of NF-κB may be responsible for NO sustained catabolic processes that lead to cartilage resorption in arthritic diseases (Clancy *et al.*, 2004). The ability of Win-55,212 to block NO production implies that this drug may also be capable of modulating the signalling pathways downstream of IL-1, including the activation of NF-κB. In this study, Win-55,212-2 appeared to reduce NF-κB nuclear translocation stimulated by IL-1 (Fig. 7.3). This could be partly through its ability to abrogate NO production but also could be partly through direct effects on the NF-κB activation mechanisms. It is possible that the effects produced by the cannabinoids are partly through their capability to attenuate NF-κB activation.

Cannabinoids have been shown to inhibit significantly NO production as well as block iNOS expression in chondrocytes (Chapter 4). The transcription of iNOS has been reported to be partially under the control of cAMP-dependent NF-κB (Berdyshev, 2000). Win-55,212-2 appeared to reduce activation of the transcription factor (Fig. 7.3 D), suggesting that cannabinoids may have the capacity to inhibit activation of NF-κB in chondrocytes. It has been suggested therefore that cannabinoids might be regulating the NF-κB-dependent iNOS transcription via cannabinoid receptor-mediated downregulation of PKA activity (Berdyshev, 2000).

It is possible that the cannabinoids are producing the cartilage protective effects via cannabinoid receptor(s). The cannabinoid receptor inactive enantiomer, Win-55,212-3, did not inhibit NO production and did not significantly protect cartilage from degradative effects of IL-1, although it slightly inhibited collagen degradation at 10 μ M. The stereoselectivity suggests that Win-55,212-2 could be acting through some receptor(s). The bovine articular chondrocytes used were shown, for the first time, to constitutively express both CB₁ and CB₂ cannabinoid receptors (Figure 7.4-7.6). Since Win-55,212-2 is a non-specific cannabinoid receptor agonist, it is therefore not possible to tell exactly at which receptor it is acting without carrying out further studies. The use of AM 281 and AM 630 (CB₁ and CB₂ cannabinoid receptor antagonists, respectively) did not help solve this dilemma because these antagonists did not antagonise but instead enhanced Win-55,212-2's effects and did not act as inverse agonists in the system tested, as would be expected.

Conclusion

Win-55,212-2 reduced IL-1 induced p38 MAPK and NF- κ B activation and this may be responsible for the other effects of Win-55,212-2 observed. It has been shown also, for the first time that chondrocytes express both CB₁ and CB₂ cannabinoid receptors. Although the chondrocytes express cannabinoid receptors, it is still not yet clear which receptor the cannabinoids may be acting through. As suggested earlier, there is a possibility that cannabinoids such as Win-55,212-2 may have another target other than the two known cannabinoid receptors.

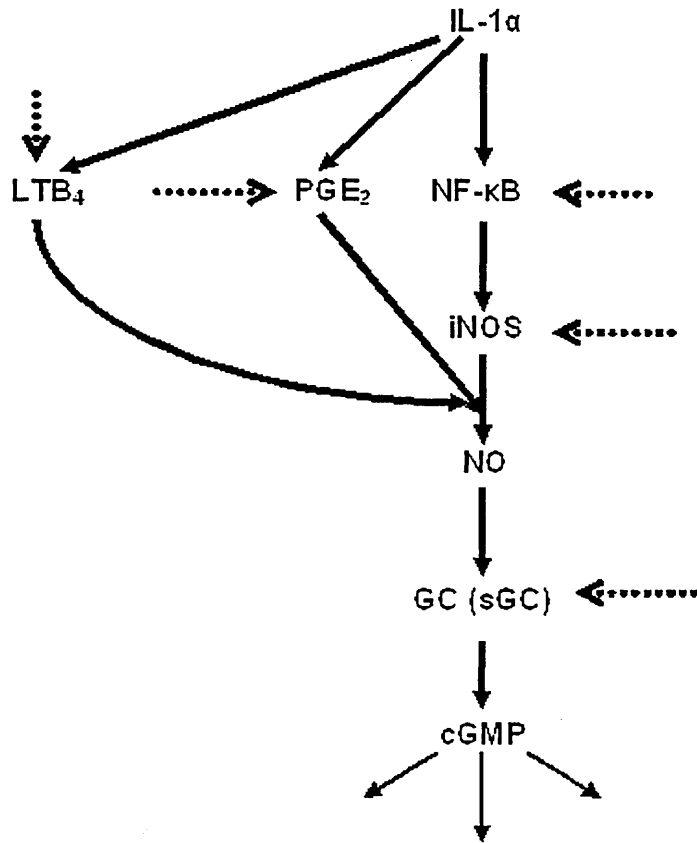
CHAPTER 8

GENERAL DISCUSSION AND SUMMARY

Cannabinoids and IL-1 α -induced NO production in chondrocytes

IL-1 stimulates the production of NO in bovine articular chondrocytes. Cannabinoids generally appeared to have the ability to inhibit NO production induced by IL-1 α at concentrations that were not toxic, in bovine articular chondrocytes (Chapter 4). Figure 8.1 below shows the proposed IL-1-induced NO production pathway in these cells and possible targets for cannabinoids. IL-1 has been shown to stimulate activation of NF- κ B, which translocates to the nucleus (Section 7.3.2) and activates transcription of a number of genes, one of which is iNOS (Berdyshev, 2000). In this study PDTC, inhibitor of NF- κ B, significantly inhibited NO production in chondrocytes, suggesting that NF- κ B activation enhances activation of iNOS and consequently NO production in these cells. Win-55,212-2 appeared to reduce the transcription factor's activation and its effects on NO and PGE₂ were similar to those of PDTC. Cannabinoids attenuated NO production partly through their ability to block iNOS expression as shown by the effect Win-55,212-2. NO activates NO-sensitive soluble guanylyl cyclase (sGC) which leads to increased production of the second messenger, cGMP. This cGMP may be responsible, at least in part, for some of the effects of IL-1 observed in chondrocytes and cartilage. The inhibition of sGC by ODQ, for instance, significantly inhibited PGE₂ and LTB₄ production in chondrocytes. Cannabinoids, therefore, by inhibiting NO production inhibited sGC activation and its subsequent production of cGMP. In summary, Cannabinoids interfered with NF- κ B activation, iNOS expression and subsequent NO production which led to inhibition of activation of sGC and subsequent cGMP production in chondrocyte stimulated by IL-1.

Inhibition of PGE₂ (using NS-398, COX-2 inhibitor) and LTB₄ (using MK-886, 5-LOX activating protein – FLAP inhibitor) production also inhibited NO production in chondrocytes indicating cross-talk (Chapter 4). The possibility of cannabinoids inhibiting NO production through inhibition, at least in part, of the COX-2 and 5-LOX pathways may not be ruled out.



KEY
> Inhibition and possible targets for cannabinoids
 —————> Activation/stimulation

Figure 8.1: Proposed schematic representation of IL-1-induced NO production pathway in chondrocytes and possible cross-talk with PGE₂ and LTB₄ production pathways

The main reactive oxygen species (ROS) produced by chondrocytes are NO and superoxide anion (O₂⁻) that generate derivative radicals, including peroxynitrite (ONOO⁻) and hydrogen peroxide (H₂O₂). ONOO⁻ is one the radicals thought to be responsible for the cell apoptosis that leads to cartilage degradation (Henrotin *et al.* 2003). Cannabinoids may also be working as antioxidant thus protecting cartilage degradation.

Cannabinoids and eicosanoid production in chondrocytes

Cannabinoids had varied effects on eicosanoids, PGE₂ and LTB₄ production in bovine articular chondrocytes stimulated with IL-1. Whilst PGE₂ was significantly enhanced by the cytokine, LTB₄ production appeared to be reduced by it, suggesting that IL-1 is not one of the factors that induce LTB₄ production in chondrocytes (Chapter 5). Some of the cannabinoids tested significantly reduced PGE₂ production induced by IL-1. This was partly due to specific blockade of expression of COX-2, since COX-1 and mPGES-2 which are constitutively expressed were not significantly affected by Win-55,212-2. However some of the cannabinoids enhanced PGE₂ production. Again some of the cannabinoids reduced LTB₄ production whilst some enhanced its production. Win-55,212-2 reduced both eicosanoids showing a high potential as an anti-inflammatory agent.

IL-1 treatment of chondrocytes has been shown to result in a dramatic dose-dependent increase in intracellular phospholipase A₂ (PLA₂) activity and subsequent secretion of this enzyme extracellularly (Gilman *et al.*, 1987). This is followed by an enhanced release of free arachidonic acid. The arachidonic acid liberated was subsequently metabolized exclusively to PGE₂; no significant increases in the production of other eicosanoids were observed including LTB₄ (Gilman *et al.*, 1987). However, soluble PLA₂ has also been shown to correlate with augmentation of LTB₄ secretion at inflammatory sites (Lam *et al.*, 1990). This may possibly be through stimulation of the PLA₂ pathway via factors other than IL-1. In an arthritic tissue, the inhibition of both the COX-2 pathway and the 5-LOX pathway may thus prove to be therapeutically more beneficial than blockade of one of the pathways (Laufer, 2001).

IL-1 stimulated production of PGE₂ in bovine articular chondrocytes (Chapter 5) and Figure 8.2 below, shows the proposed IL-1-induced PGE₂ production pathway in these cells and possible targets for cannabinoids' action. IL-1 activates PLA₂ which liberates arachidonic acid from phospholipids in the plasma membrane. COX-2 utilises arachidonic acid to produce PGG₂

followed by PGH₂. Then mPGES utilises PGH₂ to produce PGE₂. Cannabinoids have been shown in this study, to inhibit COX-2 expression and subsequent PGE₂ production. They may also significantly reduce the production of PGE₂ by inhibiting the mPGESs, especially the inducible mPGES-1.

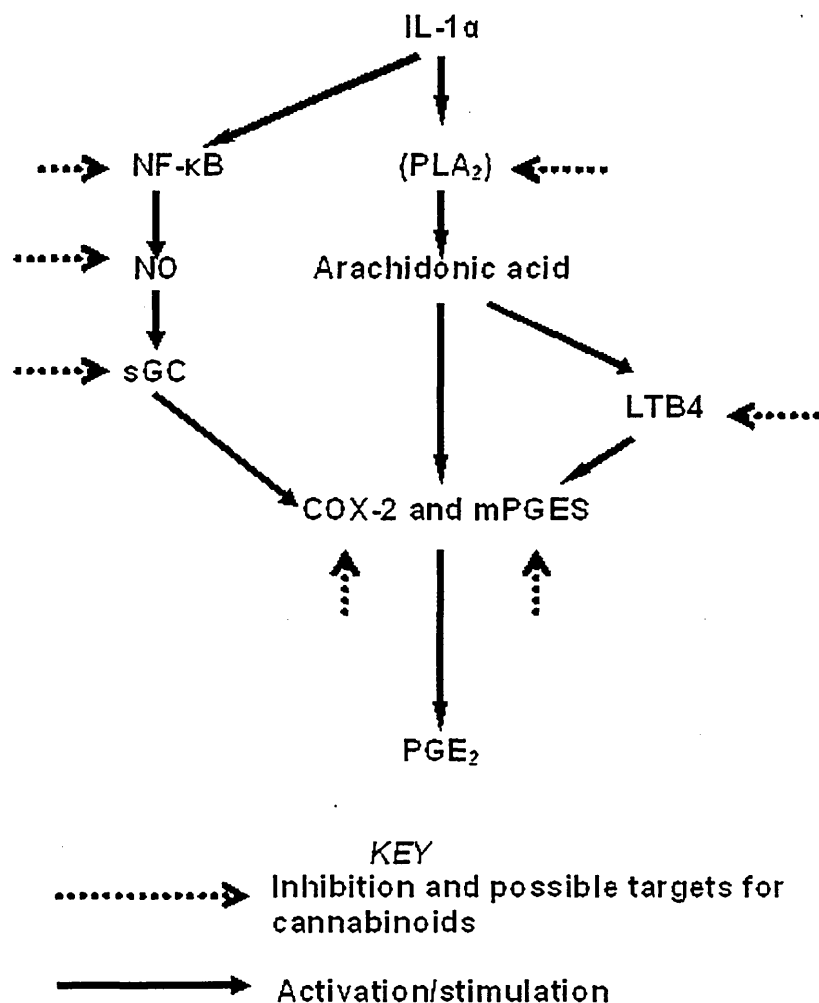


Figure 8.2: Proposed schematic representation of IL-1-induced PGE₂ production pathway in chondrocytes and possible cross-talk with NO and LTB₄ production pathways

Inhibition of 5-LOX activating protein (FLAP) by MK-886 also inhibited PGE₂ production, suggesting cross-talk between these two pathways. Inhibition of PGE₂ production by cannabinoids may possibly, in part, be through their ability to reduce LTB₄ production. IL-1 has also been shown to activate NF- κ B mediated PGE₂ production through expression of COX-2 and mPGES (Catley

et al., 2003). Since the cannabinoid Win-55,212-2 has been shown to inhibit activation of NF- κ B in chondrocytes in this study (Chapter 7), it is possible that some of the effects of cannabinoids shown on PGE₂ production were due to NF- κ B inhibition. Inhibition of NO-sensitive GC also inhibited PGE₂ production, another indication of possible cross-talk between PGE₂ and NO production pathways. Thus inhibition of NO production by cannabinoids may, in part, inhibit PGE₂ production.

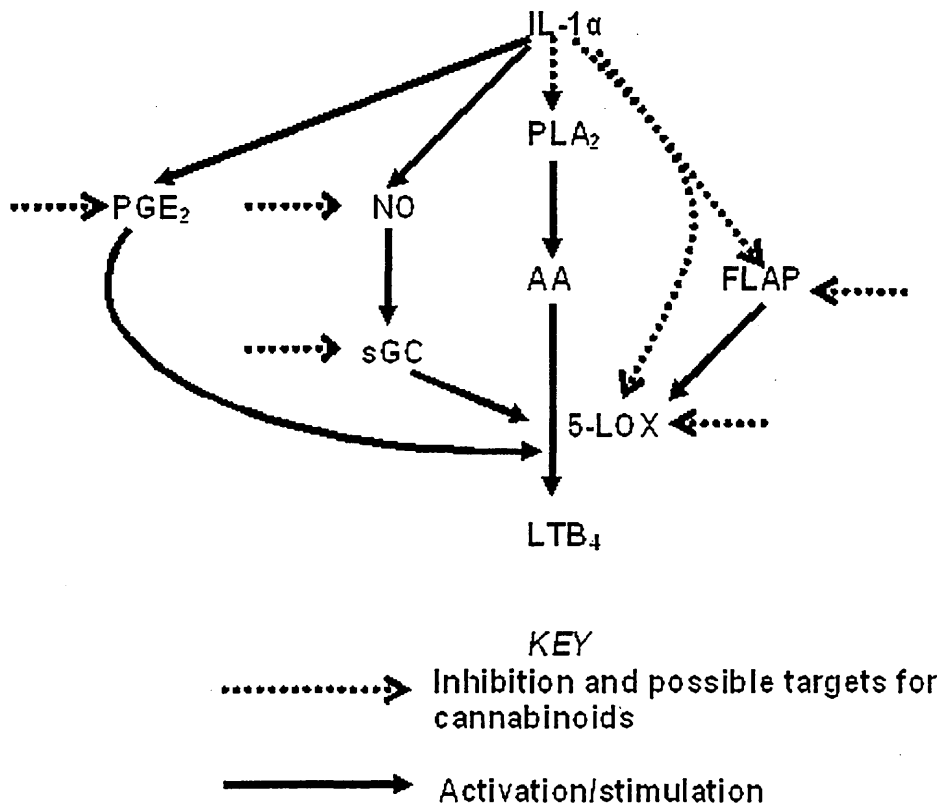


Figure 8.3: Proposed schematic representation of induced LTB₄ production pathway in chondrocytes stimulated with IL-1 α and possible cross-talk with PGE₂ and NO pathways

In bovine articular chondrocytes, IL-1 was shown to inhibit production of LTB₄ (Chapter 5) and Figure 8.3 shows the proposed LTB₄ production pathway in bovine articular chondrocytes stimulated with IL-1. It is possible that IL-1 inhibited 5-LOX or its activating protein (FLAP), thus blocking LTB₄

production. There appears to be cross-talk between the LTB₄ pathway and NO and PGE₂ pathways since inhibitors of NO-sensitive GC, ODO and inhibitor of COX-2, NS-398 reduced LTB₄ production (Chapter 5). Cannabinoids also inhibited LTB₄ production. This could be due to their ability to block the NO and PGE₂ pathways or through direct effects on FLAP and/or 5-LOX.

Cross-talk between the iNOS and COX pathways has been reported by a number of researchers (Kanematsu *et al.*, 1997; Manfield *et al.*, Uno *et al.*, 2004), suggesting that activation of one pathway modulates the other pathway negatively or positively. This implies that pharmacological agents designed to inhibit iNOS or COX-2 may influence both pathways (Legrand *et al.*, 2001). The present study also suggests that the 5-LOX pathway is also involved in the cross-talk. Therefore pharmacological agents that inhibit all three pathways may be more effective than those that inhibit just one of the pathways. Since cannabinoids, such as Win-55,212-2 exhibited the capacity to inhibit all three, COX-2, iNOS and 5-LOX pathways, this places them in a favourable position as possible therapeutic agents in arthritis.

Cartilage protection from cytokine-induced degradation

Cannabinoids were found to be able to inhibit IL-1-induced extracellular matrix degradation in bovine nasal articular cartilage. Cannabinoids blocked the induced release of COMP and significantly inhibited proteoglycan and collagen breakdown. Win-55,212-2 blocked IL-1-stimulated matrix degradation completely, allowing only basal degradation. This could be partially due to cannabinoids' ability to block iNOS, COX-2 and LTB₄ production as well as inhibition of p38 MAPK and NF-κB activation.

Possibility of existence of additional cannabinoid receptor(s)

This study has shown for the first time that chondrocytes express both CB₁ and CB₂ cannabinoid receptors. To test if the effects of cannabinoids were mediated through receptors, enantiomeric pair, Win-55,212-3 and Win-

55,212-2 was used to check for the stereoselectivity of the effects of the cannabinoids. Unlike the high-affinity cannabinoid receptor agonist Win-55,212-2, the low-affinity enantiomer Win-55,212-3 did not exert any significant inhibitory effects on the parameters tested: NO production (Chapter 4) and extracellular matrix degradation (Chapter 6). This enantiomeric stereo-specificity suggests that effects of Win-55,212-2 were receptor mediated. Since Win-55,212-2 is a non selective cannabinoid receptor agonist, selective antagonists/ inverse agonists, AM 281 and AM 630 at CB₁ and CB₂ receptors, respectively, were used to check the involvement of these receptors in the effects exhibited by Win-55,212-2. Both antagonists did not block the effects of Win-55,212-2, suggesting that effects of Win-55,212-2 were mediated by neither CB₁ nor CB₂ receptor. These findings imply presence of another cannabinoid receptor yet to be identified in chondrocytes. A number of researchers have suggested the possibility of existence of new cannabinoid receptor(s). Facchinetti *et al.* (2002) showed the stereoselectivity of Win-55,212 effects in microglial cells as well as inability of selective antagonists of CB₁ (SR141716A and AM251) and CB₂ (SR144528) cannabinoid receptors to affect the effect of Win-55,212-2, suggesting the existence of yet unidentified cannabinoid receptor(s) in brain microglia. Also anandamide and Win-55,212-2 were shown to be active in CB₁ knockout mouse brain membranes via a common G protein-coupled receptor with a distinct CNS distribution, also implying the existence of an unknown cannabinoid receptor subtype in the brain (Breivogel *et al.*, 2001). Although anandamide and vanilloid agonists share pharmacological effects, the actions of anandamide in CB₁ knockout mice appeared not to be mediated by vanilloid VR(1) receptors (Wiley and Martin, 2002). While not yet conclusive, these results suggest the possibility of additional cannabinoid receptor(s) in the brain and periphery.

Win-55,212-2 appeared to be the most efficacious cannabinoid agonist in the present studies on chondrocytes and cartilage. Win-55,212-2 high potency has also been reported elsewhere (Breivogel *et al.*, 2001) and it has been proposed that this may be explained by the existence of this extra G protein-coupled target for Win-55,212-2.

For a summary of effects of cannabinoids as observed using the synthetic cannabinoid, Win-55,212-2 at concentration that were not cytotoxic, please refer to the Table 8.1 below.

Table 8.1: Summary of the effects of Win-55,212-2

Bovine articular chondrocytes stimulated by IL-1 α (0.06 nM)

Parameter	Effect
NO production	significantly inhibited
iNOS expression	blocked
PGE ₂ production	significantly inhibited
COX-1 expression	unaffected
COX-2 expression	blocked
mPGES expression	slight inhibition
LTB ₄ production	inhibited
Phosphorylation of p38 MAPK	inhibited
Total p38 MAPK	no effect
NF- κ B activation	inhibited
Proteinase expression	reduced

Bovine nasal cartilage explants stimulated with IL-1 α (0.3 nM)

Parameter	Effect
Collagen breakdown	significantly inhibited
COMP release	blocked completely
Proteoglycan breakdown	significantly inhibited

Further work

Further studies are required to allow a full understanding of the effects of cannabinoids on chondrocyte metabolism and whether they have potential as anti-arthritic drugs. Suggestions for future work include:-

- studies of the effects of cannabinoids on IL-1-stimulated human chondrocytic cell lines or primary human chondrocytes/explants derived from human cartilage, though tissue supply may be problematic;
- studies of the effect of cannabinoids on cartilage and synovium co-cultures unstimulated or stimulated with IL-1, studying parameters such as sGAG release, hydroxyproline release and production of pro-inflammatory cytokines such as TNF;
- further studies of cannabinoid receptor expression on chondrocytes in order to determine the receptors involved in the observed actions of cannabinoids including:-
 - receptor binding studies;
 - studies on receptor knockdown using siRNA and its effects on cannabinoid action;
- determination of the effect of cannabinoids on the expression of specific proteinases such as MMP-1,-2,-3 and -9 and aggrecanases as well as expression of inhibitors of these proteinases in cartilage explant and chondrocyte cultures.

These studies would help to further evaluate the role of cannabinoids in preventing cartilage breakdown.

Conclusion

It would be worthwhile to consider Win-55,212-2 and other cannabinoids with similar potency and efficacy for further studies or trials in arthritic disorders. The use of nonpsychoactive cannabinoids such as ajulemic acid, cannabidiol and dexamabinol as well as CB₂ cannabinoid receptor agonists may allow the dissociation of unwanted psychoactive effects from potential therapeutic benefits. The existence of other cannabinoid receptors may provide potential therapeutic targets that are independent of CB₁ receptors and thus devoid of the adverse effect associated with the central cannabinoid receptors. Elucidation of the most appropriate route of delivery, eliminating smoking,

may increase the efficacy of the cannabinoids and may evade or reduce adverse effects. This thesis suggests that cannabinoids may protect cartilage from degradation, partly through their ability to modulate production of pro-inflammatory mediators, NO and PGE₂.

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PUBLICATIONS AND CONFERENCE ABSTRACTS

Publications

- I. Mbvundula EC, Rainsford KD, Bunning RA. Cannabinoids in pain and inflammation. *Inflammopharmacology*, 2004; 12(2):99-114.
- II. Estery C Mbvundula, RAD Bunning and KD Rainsford. Effects of cannabinoids on nitric oxide production by chondrocytes and proteoglycan degradation in cartilage. *Biochemical Pharmacology*, 2005; 69(4): 635-640.
- III. One more manuscript submitted for publication in the *Journal of Pharmacy and Pharmacology*

Conference Poster Abstracts

- **The effects of opioids and cannabinoids on chondrocytes**
RAD Bunning, E Mbvundula, J Parker, R Seabrook and KD Rainsford

International Conference on Inflammopharmacology with VIII Side-Effects of Anti-Inflammatory Drugs Symposium, 22-24 April 2003, Royal College of Physicians of Edinburgh, Edinburgh, UK
- **The effects of cannabinoids on IL-1 α -induced cartilage resorption**
Estery C. Mbvundula, Rowena A.D. Bunning and K.D. Rainsford

The XIXth Federation of European Connective Tissue Societies (FECTS) Meeting, 9-13 July 2004, Italy
- **R-(+)-Win-55,212-2 inhibits nitric oxide and prostaglandin E₂ production and cartilage degradation**
Estery C Mbvundula, RAD Bunning and KD Rainsford

BioScience2004 – from molecules to organisms, The Biochemical Society International Conference, 18-22 July 2004, SECC, Glasgow, UK
- **Cannabinoids in contrast to opioids may be cartilage protective via inhibition of cytokine-induced nitric oxide and prostaglandin E₂ production and cartilage degradation**
EC Mbvundula, RAD Bunning, KD Rainsford, J Parker and R Seabrook

