Magdalena Krasimirova Kaneva

Investigation into the immuno-therapeutic potential of melanocortin peptides on activated chondrocytes

This thesis is presented for the degree of Doctor of Philosophy of University of Westminster
2011

Department of Human and Health Sciences
School of Life Sciences
University of Westminster
London, United Kingdom
DECLARATION

I declare that this thesis is the result of my own research, and as its main content it contains work that has not been previously submitted for degree at any tertiary educational institution.

Magdalena K. Kaneva
I dedicate this Ph.D. dissertation to my teachers and parents

Посвещавам тази докторска дисертация на моите учители и родители
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ABSTRACT

Melanocortin peptides are endogenously produced peptides originating from the post-translational processing of the pro-opiomelanocortin hormone (POMC), exerting their effect by binding to class A G-protein-coupled 7 transmembrane domain receptors, positively coupled to adenylate cyclase. To date five melanocortin receptors have been identified and termed MC1 to MC5. MC1 and MC3 have previously been proposed to exert anti-inflammatory effects by modulating the host inflammatory response. The expression and the functional activity of both receptors was identified and confirmed in the C-20/A4 chondrocyte cell-line, isolated primary bovine and in situ bovine articular chondrocytes.

Pro-inflammatory cytokines including IL-1β, IL-6, IL-8, TNF-α, produced by activated articular chondrocytes significantly up-regulate matrix metalloproteinases (MMPs) gene expression, and inhibit the chondrocyte’s compensatory synthesis pathways required to restore the integrity of the degraded extracellular matrix (ECM). Human C-20/A4 and primary bovine articular chondrocytes were found to produce CC and CXC chemokines, which induced the release of matrix degrading enzymes and activated cell apoptotic pathways. TNF-α significantly up-regulated the expression of pro-inflammatory cytokines and chemokines IL-1β, IL-6, IL-8, MCP-1 and MMP1 and 13 from C-20/A4 cell line and freshly isolated primary bovine articular chondrocytes. An effect attenuated in the presence of α-MSH and D[TRP]8-γ-MSH. The MC3/4 antagonist SHU9119 blocked the effects of D[TRP]8-γ-MSH but not α-MSH.

TNF-α (60.0 pg/ml) stimulation caused ~30% cell death and was partially, but significantly inhibited by treatment of the cells with the melanocortin peptides. The anti-inflammatory and chondroprotective effect of melanocortin peptides were then tested on in situ bovine articular chondrocytes, injured by a single blunt impact delivered by a drop tower. The mechanical injury caused significant cell death and up-regulation of the pro-inflammatory cytokines IL-6 and IL-8, which were significantly reduced on pre-treatment of cartilage explants with melanocortin peptides.

Modulation of pro-inflammatory pathways and inflammation-modulated cartilage destruction with subsequent chondrocyte apoptosis appears to be logical development in the potential medical therapy of OA. The small molecular weight of melanocortin peptides should facilitate the absorption from the GI tract and the movement to the cartilage matrix, which together with creative drug delivery methods might potentially prove to be potent therapeutic agents in the future.
ACKNOWLEDGEMENTS

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<td>AA</td>
<td>Acrylamide</td>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic Hormone</td>
</tr>
<tr>
<td>Anti-CCP</td>
<td>Anti-Cyclic Citrullinated Peptide</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating Protein-1</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-Triphosphate</td>
</tr>
<tr>
<td>A_{xxxnm}</td>
<td>Absorbance at xxx nm</td>
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<tr>
<td>BA</td>
<td>Bis–Acrylamide</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<td>BSA</td>
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<td>Aspartate residue</td>
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<td>Dithiotreitol</td>
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<tr>
<td>ECSIT</td>
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</tr>
<tr>
<td>EDTA</td>
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<td>EIA</td>
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<td>ELISA</td>
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<tr>
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<td>Extracellular signal-regulated kinase</td>
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<td>mAb</td>
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<td>MAPK</td>
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</tr>
<tr>
<td>pAb</td>
<td>Polyclonal Antibody</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PGD₂</td>
<td>Prostaglandin D₂</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PGG₂</td>
<td>Prostaglandin G₂</td>
</tr>
<tr>
<td>PGH₂</td>
<td>Prostaglandin H₂</td>
</tr>
<tr>
<td>PGES</td>
<td>Prostaglandin E Synthase</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric Point</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid Factor</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor Interacting serine-threonine Protein kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SBE</td>
<td>STAT-Binding element</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Means</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris Borate Ethylene–(2,2’)-diamine–tetracetic acid</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethyl-1,2-diaminoethane</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane Domain</td>
</tr>
<tr>
<td>TNF–α</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF-α Receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF-α Receptor Associated Death Domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF-α Receptor Associated Factor</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<td>-------------</td>
</tr>
<tr>
<td>TYK2</td>
<td>Tyrosine Kinase-2</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>α-MSH</td>
<td>Alpha–melanocyte stimulating hormone</td>
</tr>
<tr>
<td>β-MSH</td>
<td>Beta–melanocyte stimulating hormone</td>
</tr>
<tr>
<td>γ2–MSH</td>
<td>Gamma–2 melanocyte stimulating hormone</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
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PUBLICATIONS

Papers


Abstracts


KANEVA, M., KERRIGAN, M., LOCKE, I. C. & GETTING, S. 2009. Pro-inflammatory mediator release from TNF-α stimulated C-20/A4 cells results in Col II degradation. BORS, Newcastle, PO136

Chapter 1
Introduction
1.1 Cartilage.

Mammalian cartilage is a complex and developmentally critical tissue that has been historically defined as a vertebrate tissue component of the skeletal system, fundamentally different in structure and function from bone tissue. It functions as an internal cellular support tissue and is composed of fibrous proteins and mucopolysaccharides (Cole and Hall, 2004). Three different types of cartilage exist in mammals; each differing in the arrangement and proportion of extracellular fibrous (collagen) or non-fibrous (proteoglycans) macromolecules within the extracellular matrix (ECM), in the distribution and morphology of the only resident cell type – the chondrocyte, and their anatomical location and functions within the body (Hall, 1998).

Hyaline cartilage, also wildly known as articular cartilage, is the most studied cartilaginous tissue. It is particularly important due to its function in development, as it lays down the scaffold for the future development of most of the key bones within the body, and due to its preservation at the joint interfaces, where it creates a smooth surface for frictionless joint movement. It is also the primary tissue affected by arthritic pathologies (Hollander et al., 2010).

In hyaline cartilage, the proportion of collagen to proteoglycans, is such that under polarized light microscope the tissue appears glassy, with translucent blue-white appearance in early age, turning pale and opaque with aging (Van der Korst et al., 1968, Hall, 1998). Articular cartilage constitutes the temporary embryonic skeleton, before its replacement with bone. It can also be found in the epiphyseal plates between the diaphysis and epiphysis of growing long bones, where it is responsible for the longitudinal growth of the bone (Martel-Pelletier and Pelletier, 2010). In adults, this type of cartilage is present in the articulating surfaces of articular joints, in the wall of the large respiratory passages, such as the nose, larynx, trachea, bronchus, and at the ventral ends of the ribs that move with the sternum. The tissue is avascular, alymphatic and aneural and populated only by chondrocytes, which are responsible for the synthesis, exportation and degradation of ECM components – proteoglycan, fibrous collagen (type I, type II, and small amounts of types VI, IX and XI) and other proteins. The surface areas of the articular cartilage can withstand tensile forces arising from joint movement.
but its principle adaptation is to withstand compressive forces (Martel-Pelletier and Pelletier, 2010).

Fibrocartilage, or white fibrocartilage, is another type of cartilage found mainly in the invertebrate disks of the spine, the attachment of some ligaments to the bones, in the pubic symphysis, but also in knee, jaw and healing bones (Benjamin and Evans, 1990, Benjamin and Ralphs, 1998). Its main function is to withstand compression forces. Fibrocartilage is a very dense connective tissue, composed mainly from collagen type I (Eyre and Muir, 1975), relatively little proteoglycan (Gillard et al., 1979, Koob and Vogel, 1987) and some elastic material (Buckwalter et al., 1976). It develops by metaplasia from pre-cartilage, hyaline cartilage and particularly from fibrous tissue, and these features precede the appearance of cartilage cells (Benjamin and Evans, 1990).

The third type of cartilage is the elastic cartilage, or yellow fibrocartilage. Elastic cartilage is composed of elastic fibres as well as collagens and proteoglycans and is mainly found in the auricle of the ears, in the Eustachian tubes and epiglottis. It is described as rubber-like, highly and reversibly deformable. Elastic cartilage chondrocytes highly resemble those in hyaline cartilage, but these synthesize elastin in addition to the other matrix components (Hall, 1998).

1.1.1 Articular Cartilage – structure and function.

The mechanical resistance and flexibility of cartilage occurs because of the four components of the extracellular matrix and their properties – proteoglycans, collagen, interstitial fluid and chondrocytes. Proteoglycans are proteins composed of highly negatively charged glycosaminoglycans (GAGs), covalently attached through O-glycosidic bond at their reducing ends to the core protein, commonly chondroitin sulphate (CS) or keratan sulphate (KS) (Jackson et al., 1991). Aggrecan, one of the largest proteoglycans and the major one in articular cartilage, is a large chondroitin sulphate proteoglycan composed of a large number of GAG monomers and represents the bulk of the proteoglycans in cartilage (Watanabe et al., 1998). It interacts with the hyaluronan and other link proteins to form macromolecular complexes, trapped by the collagen network and gives cartilage its capacity to resist compression through hydration (Jackson
et al., 1991). Decorin, biglycan and fibromodulin, are smaller proteoglycans, rich in leucine, representing around 20-25% of all proteoglycans in cartilage, and are associated with the proteoglycans and the ‘ground substance’ – the main components of which are large carbohydrates and other proteins (Hardingham and Fosang, 1992). A central component of the proteoglycan aggregates is hyaluronan, which is a non-sulphated glycosaminoglycan and is present in relatively small amounts in cartilage. It is, however, an important component of the matrix, as CD44, expressed on the chondrocytes cell surface, bind to hyaluronan, thus embedding the chondrocyte within the extracellular matrix (Figure 1.1).

Collagen fibres are the principle component of connective tissues and are composed of bundles of fibrils, which are made of stacked molecules of helical polypeptide chains. Fourteen different types of collagens exist, which vary both in size and in configuration (Eyre, 2004). Articular cartilage is mainly composed of a unique type of collagen, type II, which is structurally characterised by the aggregation of large fibrillar homotrimer complexes, with three identical α-chains peptides (formed by the repeating tri-peptide sequence Gly-x-y) each adopting left-handed helical conformation (Hall, 1998) with the three chains twisted together in a right-handed helix. Collagen type II in cartilage forms a 3D fibrillar network of rope-like molecular aggregates, which together with the proteoglycans is essential for maintaining the tissue volume, shape and tensile strength (Eyre et al., 1992). Collagen II represents >95% of all the collagens present in the cartilage. Other collagens found in cartilage include type I, type IX, type X, which play an essential role in the matrix organization. Collagen type IX is a non-fibril, highly glycosilated collagen that covalently links to type II collagen fibrils, thereby helping in the interweaving and cross-linking of the collagen fibrils and trapping the hydrated proteoglycans. Collagen type IX is additionally regarded as a proteoglycan due to a CS chain attached and is thought to provide lateral strength and flexibility (Hall, 1998).
Figure 1.1 Articular cartilage zones and zonal orientation of collagen type II fibrils.
A simplified diagram of a perpendicular section of articular cartilage and underlying bone, showing the heterogeneity of the cartilage matrix. Arcades of collagen bundles originating in the calcified zone and binding the non-calcified cartilage are extending towards the articular surface and then curving back down. In sequence from the articular surface to the subchondral bone, the collagen bundles are tangential, radial and perpendicular. The collagen fibres are continuous with those in the calcified layer of cartilage but not with underlying subchondral bone. The morphology of the collagen-fibril network influences the local stresses and strains in the articular cartilage.

The organisation and distribution of ECM macromolecules and chondrocytes differs significantly within the tissue and can be separated into four distinct regions. The first region located at the articular surface of the cartilage is the superficial zone. It is composed of thin, tangential, tight collagen fibrils, mainly
parallel to tangential stress and is associated with low concentration of aggrecan and high concentrations of decorin. The middle zone contains thick radially, randomly oriented, looser bundles of collagen, whilst the deep zone (also called radial zone) contains perpendicularly oriented collagen fibrils and the calcified cartilage zone (located between the deep zone and the subchondral bone) serves as a mechanical buffer (Muir et al., 1970, Wilson et al., 2004). It has been observed that the cell density progressively decreases from the superficial zone to the deep zone, whereas relative to collagen, cell volume and the proteoglycan content increase.

Within cartilage, there are not only topographical variations in the quantity of proteoglycan, but also the type, thus contributing to the physical properties of the matrix. The GAG side chains of proteoglycans are sulphated and responsible for a fixed negative charge density, which binds mainly sodium, but also other mobile cations (Lesperance et al., 1992). This process, together with the quality and quantity of the collagen network is deterministic for the osmotic pressure of the extracellular fluid around chondrocytes. Extracellular osmolarity in healthy articular cartilage ranges between 350 and 480 mOsm.kg\(^{-1}\) H\(_2\)O, hereafter termed ‘mOsm’ (Maroudas, 1976) and is highly dynamic, in response to changes in matrix hydration (Urban, 1994). During osteoarthritis, the collagen matrix degrades and the concentration of GAG diminishes in a disease progression-dependent way, leading to decreased osmolarity to between 280-350 mOsm (Maroudas, 1976, Bush and Hall, 2001a, Bush and Hall, 2005).

The proteoglycan molecules (3–10 % of tissue on wet weight basis) are highly negatively charged with sulphate, SO\(_4^{2-}\) and carboxyl (COO\(^-\)) functional groups, which, together with the positively charged cations drawn from the synovial fluid, cause the osmotic pressure in the matrix to increase and consequently leads to water inhibition and tissue inflation. However, within the cartilage, the expansion of the proteoglycans is limited by the tension of the collagen matrix (15 – 30 % of tissue on wet weight basis), which restricts proteoglycans hydration to only 20 % of their actual capacity, and thus creates swelling pressure within the cartilage, contributing to its compressibility (Hall, 1998). When cartilage is loaded, during normal daily activities, water is extruded and the PGs are further compressed, but upon removal of the load (resting) water is imbibed in the tissue, together with
essential nutrients, until the swelling pressure of the aggregcan is balanced again by the tensile force of the collagen matrix. The interstitial fluid (65 – 80 % of the tissue) is what forms the aqueous environment in which the chondrocytes reside. It supplies the cells with substrates, nutrients and matrix biosynthesis precursors, and also allows for metabolic by-products to be eliminated from the tissue.

1.1.2 Chondrocytes.

Connective tissues are highly active living tissues with capacity to support and resist mechanical forces, crucial for the musculoskeletal system. The cells of connective tissues continually produce and maintain appropriate macromolecules that give the tissue its required mechanical properties. This occurs by synchronised equilibrium of anabolism and catabolism of those macromolecules (Gardner, 1992).

Chondrocytes are present at a very low density of ~ 1-5 % of the adult cartilage tissue, with approximately 1.0 x 10^3 cells per mm³ (human femoral head), and depending on the location, little or no cell division (Hall, 1998). Although the role of the chondrocytes has historically been marginalized because they do not play a direct mechanical role, they are the only existing units capable of producing, repairing or degrading the matrix. Additionally, chondrocytes ‘sense’ physical changes in their environment and this determines matrix metabolism and ultimately the mechanical characteristics of the tissue, the osmotic composition of the interstitial fluid, cell-matrix interactions and the rate of diffusion of substances between the synovial fluid and the interstitial fluid.

Chondrocytes in cartilage are heterogeneous, and this is related to the topography within different zones of the tissue (Stockwell and Meachim, 1973, Hall, 1998). In addition chondrocytes can exist as single units or in groups of up to six cells. Differences in the morphology of zonal chondrocytes can be easily observed. Chondrocytes from the superficial zone occur singly or in pairs, are small (8–15 µm in diameter), sparsely distributed and ellipsoid in morphology. In the middle zone, chondrocytes are oval-shaped approximately 15–25 µm in diameter and are arranged radially, singularly or in pairs (Stockwell, 1971, Hall et al., 1996, Hall, 1998). Chondrocytes become more oval in the deep zone, where
they are found in small groups or short columns of about 3–5 cells (Hall, 1998, Hall et al., 1996).

Early studies have identified that the matrix immediately surrounding the chondrocyte in hyaline cartilage is different and more specialised than the intercellular/interterritorial matrix, which led to the identification of a primary functional and metabolic unit termed ‘chondron’ (Poole et al., 1992). The chondron consists of one or several chondrocytes, the pericellular, or “lacunar” matrix, which is rich in proteoglycans and possibly hyaluronic acid, relatively no collagens, and the pericellular rib, which includes types VI, II and IX collagen (Figure 1.2). It has been hypothesized that the microenvironment, surrounding the chondrocytes within their chondrons, serves to protect the chondrocytes by dampening the physicochemical, osmotic and mechanical changes that happen during dynamic loading (Poole et al., 1992). Continuous with the pericellular matrix is the territorial matrix, which is composed of basket-like network of cross-linked collagen fibrils, forming a capsule around the chondron, which the chondrocyte contacts through numerous cytoplasmic processes, rich in microfilaments. The pericellular collagens diverge and converge at the superficial pole of the chondron capsule, forming a pericellular channel between the interior of the chondron and the intercellular matrix. It has been suggested that, during compressive pressure to tissue, these “pericellular channels” underline a mechanism for the chondrocytes to sense changes in the osmotic pressure and therefore regulate matrix metabolism (Hall et al., 1996).
Figure 1.2 High power phase contrast confocal micrograph of articular hyaline cartilage excised from adult (18-24 months old) bovine ‘knee’ joint.

Articular cartilage has a complex internal structure. Composed of four poorly demarcated zones, the most superficial, uppermost zone forms the gliding surface and is in contact with the synovial cavity of the joint. Small elliptical chondrocytes are oriented parallel to the surface; chondrocytes in deeper zones are larger, more rounded, and arranged in vertical columns. The term chondron encompasses the chondrocyte and its pericellular and territorial matrix. Image was taken using Leica TCS SP2, x630 magnification.
1.2 Arthritides.

1.2.1 Rheumatoid Arthritis (RA).

Rheumatoid arthritis is a complex systemic, inflammatory pathology, primarily affecting the joints, with cardiovascular problems being causal to 40% of the morbidity associated with this pathology. RA is one of the most common of the arthritides, affecting middle-aged males and females, with a marked female bias (approx. 2.5 times; (Lee and Weinblatt, 2001) as well as children (in the case of juvenile variant). Rheumatoid arthritis has acute or insidious onset, and typically, but not exclusively, affects the joints in a symmetrical manner the radiocarpal and metacarpophalangeal, proximal interphalangeal joints of the hands and feet and occasionally large joints (Lee and Weinblatt, 2001). Cardinal features of RA are early morning stiffness and pain, which can last for more than 1 h, joint swelling and deformity, synovial thickening, reduced range of motion of the affected joint and systemic features including flu-like symptoms, fatigue, fever, depression and weight loss (Lee and Weinblatt, 2001).

The exact pathology of RA is still not understood, but it is generally accepted to be a disease of autoimmune origin, resulting in a continual immunological reaction, mostly directed against joint tissues, but may also have manifestations as a more systemic syndrome involving a variety of organs (formation of rheumatoid nodules in skin, salivary inflammation, pulmonary fibrosis, pericardial inflammation, myocarditis, mononeuritis, anemia, thrombocytosis, vasculitis (Lee and Weinblatt, 2001). Characteristic findings in RA joints include synovial inflammation with an accumulation of inflammatory cells, increased synovial levels of pro-inflammatory cytokines, and the formation of a characteristic area of granulation tissue known as a pannus, which is formed from both synovial cells and the cellular infiltrate, and appears to contribute to the destruction of the underlying cartilage (Lee and Weinblatt, 2001).

Quantitative analyses have shown that a wide variety of pro-inflammatory cytokines are present in inflamed synovial tissue and are produced in moderate to high concentrations in RA. Tumor necrosis factor (TNF)-α and Interleukin (IL)-1β are both present in high concentrations in affected synovial fluid and synovial
tissue (Chu et al., 1991, Wood et al., 1992, Lee and Weinblatt, 2001), where they act as potent stimulators of proliferation, matrix metalloproteinase expression, up-regulation and release of pro-inflammatory cytokines and prostaglandin production (Lee and Weinblatt, 2001). Previous studies have identified IL-1β as the main mediator for the initiation of the proteolysis in rheumatoid arthritis and in other inflammatory joint diseases (Dayer et al., 1979, Mizel et al., 1981, Krane, 1982, Baracos et al., 1983, Wood et al., 1992). The enhanced secretion of collagenases and prostaglandin E₂ (PGE₂), by synovial cells, is considered a factor for the degenerative progression that is clinically observed. TNF-α, a potent stimulator of collagenase and PGE₂ production in synovial cells (Choi et al., 2009, Chu et al., 1991, Kunisch et al., 2009), is also implicated in the disease progression with production in far larger quantities than IL-1β under some circumstances in vivo.

Whilst RA is generally classified as an autoimmune disease, the mechanisms involved and the significance of auto-antibodies routinely detected in RA patients, remain uncertain. The main auto-antibodies, detected in roughly 70–80% of RA patients (Avouac et al., 2006), are rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP), antibodies directed against the fragment crystallizable (Fc) region of IgG antibodies and proteins containing citrulline (a modified form of the amino acid arginine), respectively (van Venrooij et al., 2008, van Venrooij and Zendman, 2008, Getting et al., 2009). It is suggested that these antibodies may be involved in RA via the formation of immune complexes, which together with their target antigens result in complement activation, the release of other pro-inflammatory mediators from the synovium, and leukocyte recruitment and infiltration into the joint space (Lee and Weinblatt, 2001, Brennan and McInnes, 2008, van Venrooij and Pruijn, 2008).

1.2.2 Metabolic Arthritis/Gouty Arthritis.

Gouty arthritis is a chronic joint disease caused by the deposition of monosodium urate monohydrate (MSU) crystals in the joints (McCarty et al., 1966, McGill and Dieppe, 1991, Chilappa et al., 2010). It is an inflammatory arthritis that predominantly affects middle-aged/older individuals, with incidence significantly higher in men than in pre-menopausal women (Getting and Perretti, 2001). Gouty
arthritis can occur in any synovial joint, but mainly manifests as a monoarthritic attack in the smaller joints, particularly the metatarsophalangeal joint, with clinical manifestation of symptoms including erythema, edema of the affected joints and severe pain (McGill and Dieppe, 1991). Gouty arthritis occurs predominantly as a consequence of hyperuricaemia (increased levels of plasma urate; a product of purine metabolism), although other factors including joint trauma and local temperature may be additionally involved (Heuckenkamp et al., 1982, Fokter and Repse-Fokter, 2010). When plasma solubility of urate is exceeded, MSU crystals deposit in a range of soft tissues and joints, resulting in gouty arthritis.

The mechanisms of urate-induced inflammation are not completely understood. Gouty arthritis seems to be initiated primarily by an innate immune response, particularly due to the intense infiltration of blood-borne neutrophils and monocytes into the joint space (Terkeltaub et al., 1991a,b; Villiger et al., 1992), resulting from the elevated secretion of pro-inflammatory mediators, such as IL-1β, IL-6, IL-8, TNF-α and thus the increased expression of endothelial cell adhesion molecules (di Giovine et al., 1991, Villiger et al., 1992). Recent work has shown that toll-like receptors TLR2 and TLR4, present on phagocytes and other cells contained in the joint environment, recognize the MSU crystals, which upon binding to the receptors, induce generation of these pro-inflammatory intermediaries, thereby providing a possible mechanism for disease instigation and progression (Cronstein and Terkeltaub, 2006).

1.2.3 Osteoarthritis (OA).

Osteoarthritis is a slowly progressive, degenerative, multifactorial disease characterized by gradual degradation and loss of articular cartilage, and resulting in loss of joint mobility and pain (Goldring, 2000a, Aigner and Stove, 2003, Goldring and Goldring, 2007). OA has been classified as a late-onset, complex disease, which affects over 100 million individuals all over the world (Iliopoulos et al., 2008). It is the most common of the arthritides, and is largely a disease of the elderly and middle aged. In theory, OA can affect any synovial joint in the body, but OA lesions are typically localized to the weight-bearing regions of the larger joints (the hip, knee and spine), and later in those of the hand (Buckwalter and Mankin, 1997).
The exact pathogenesis of osteoarthritis is not fully understood with obesity, age, abnormal joint loading and sport injuries all being risk factors (De Bri et al., 1998), but OA is more than just the result of “wear and tear” (Aigner et al., 2004b). Abnormal joint loading causes changes in physiology of the chondrocyte, which mediate the biosynthesis of ECM macromolecules. It is now generally accepted that the articular chondrocytes play an important role in the initiation and progression of OA (Goldring, 2000b).

It is not yet known what is the exact role of the chondrocyte in the initiation and progression of osteoarthritis, due to the complexity of the disease process and its high localization of focal lesions. Dieppe, 1994 hypothesized that the initiation and the progression of osteoarthritis within individual joints, and even the disease distribution between the joints, may be ascertained by the specific balance between local and systemic factors (Dieppe and Kirwan, 1994). In the cases where, for example, excessive mechanical loads cause micro-damage to cartilage, chondrocytes might not be involved directly. The injury might initiate a sequence of cartilage ECM damage, before the obvious changes to chondrocyte physiology are detected. It has been observed that there are focal defects (peak loading location) in osteoarthritis of the hip and knee, suggesting that in some cases mechanical factors are not only an important risk factor for the development of changes to chondrocyte physiology, but also a key disease initiation factor. Additionally, normal ‘wear and tear’ has been considered a significant risk factor for the development of the disease; however, the fact that the increase in middle zone tissue hydration is the first detectable change, and without any change to the collagen content, this must be resulting from weakening of the collagen network (Stockwell, 1991, Gardner, 1992). These changes vary significantly from the processes taking place during aging, whereby cartilage loses hydration and becomes ‘dry’. If the effect of the physio-chemical environment of the chondrocytes on their physiology is considered, it is possible to hypothesize that a group of susceptible chondrocytes, with changes to their physiology, start a sequence of events leading to the production of mechanically compromised matrix and predisposing cartilage to damage and degradation (Hall et al., 1996).
However, other factors are also implicated in this intricate disease process. OA is generally regarded as primarily non-inflammatory arthropathy. Nonetheless, elevation of pro-inflammatory cytokines in the cartilage of many OA patients has been detected (Westacott and Sharif, 1996, Fernandes et al., 2002) and local inflammation has been observed in animal models of the disease (Goldring, 2000a, Rai et al., 2008). Elevated levels of pro-inflammatory cytokines induce the expression and secretion of cartilage-degrading proteases by chondrocytes, leading to the degradation of cartilage ECM (Shlopov et al., 2000, Fernandes et al., 2002).

Additionally, chondrocyte apoptosis and senescence are processes that can also lead to impaired ECM synthesis (Kim et al., 2000, Aigner et al., 2004a, Roach et al., 2004, Kim and Blanco, 2007). Generally, a variety of chemical stimuli appear to promote chondrocyte apoptosis by elevating pro-inflammatory cytokines, nitric oxide (NO), and Fas ligand (FasL) production and release.
1.2.3.1 Etiology and Epidemiology of OA.

Osteoarthritis, the most prevalent musculoskeletal system disorder throughout the world, is believed to be a consequence of mechanical and biological events that destabilize the normal link of matrix synthesis and degradation within articular cartilage. OA is exceptionally common, particularly among people over 40 years of age (Guccione, 1997). It represents a major cause of morbidity, disability and social isolation, especially where the hip and knee are involved, as this can lead directly to reduced mobility (Ettinger and Afable, 1994). Radiographic changes of joints occur in the majority of people by the age of 65 and are present in more that 80 % of people over 75 years old (Arden and Nevitt, 2006). OA is a disease affecting joint cartilage and the subchondral bone. Its earliest pathologic manifestation is the degradation of cartilage, which when extensive, is visible on radiographs as a narrowing of joint space. Consequent changes in the bone include eburnation of underlying bone, osteophyte formation and sometimes formation of cysts on the subchondral bone (Martel-Pelletier et al., 1999). Additionally, there are radiographically visible lateral osteophytes, before any changes in the joint space can be detected. The disorder leads to significant increase in morbidity, especially in the elderly (Guccione, 1997), whilst osteoarthritis of the knee and hip have the highest social cost and most associated disability (Bergstrom et al., 1986).

Knee OA is very common and frequently symptomatic illness, often associated with disability, whereas hip OA is slightly less prevalent with symptoms, which are often more severe and more frequent. Knee osteoarthritis prevalence increases with age (Lawrence et al., 1966, Hernborg and Nilsson, 1973, Bergstrom et al., 1986, Felson et al., 1987) from negligible in those aged 25 – 34% to 20 – 40 % prevalence in people aged >70 years. The prevalence is higher in males than in females up to approximately 45 years old, after which the reverse is true, probably due to the inhibitory effect of estrogen on matrix metalloproteinases (MMPs) production and activation (Claassen et al., 2010). Population-based radiographic prevalence England surveys were performed for hip OA and found that radiographic OA increases with age in subjects aged 55-74 years, with 16% of men and 6% of women affected. However, other studies of Caucasian populations in Switzerland and Israel have reported equal rates in
older men and women and lower rates (ranging from 4-7 %) in general as compared to the studies in England (Lawrence and Zinn, 1970, Zinn, 1970, Danielsson et al., 1984). It has been found that race plays an important role in the incurrence of OA, with Afro-Caribbean, Chinese and Asian showing much lower rates of hip OA than Caucasian (for a review see: (Allen, 2010, Allen et al., 2010).

There are a number of possible causes for the increase of disease prevalence with age including metabolic changes occurring with ageing, which are not parallel to biochemical changes, but may render cartilage more susceptible to fatigue fractures (Claassen et al., 2011). However, there are other risk factors that have been found to correlate with the incidence of OA. Population based studies have found an association between obesity and radiographic OA of the hands, feet and knee (Denisov et al., 2010), but clinical studies evaluating this relation have not been consistent. Animal studies on rodents have demonstrated that major joint injury causes OA, whereby OA is actually induced by controlled tears of meniscus and cruciate ligament (Troyer, 1982). Such damage is often associated with damage to articular cartilage, the primary lesion of OA and changes in biomechanics, with the latter causing increased shear stress on local areas of articular cartilage. Job occupation can also influence the rate of OA incidence, with repetitive use of particular joints appearing to be a major risk factor for osteoarthritis especially of the hands (McCarthy et al., 1994, Rossignol et al., 2005, Fontana et al., 2007, Ding et al., 2010). This has also been identified within the knee (Dahaghin et al., 2009, Reid et al., 2010, McWilliams et al., 2011) and hip (Rossignol et al., 2005, Franklin et al., 2010), although the specific physical activity that causes it is not well understood. Other studies have identified other possible risk factors for osteoarthritis, such as diabetes, hypertension, cardiovascular disease, peripheral vascular disease, congestive heart failure, which have also been suggested to play a role in the initiation of osteoarthritis of knees, hips, hands and spines of patients (Felson et al., 2000, Solomon, 2001, Marks and Allegrante, 2002b, Marks and Allegrante, 2002a, Singh et al., 2002, Bray, 2004, Parada-Turska and Majdan, 2005). There is still, however, the need for new work exploring factors associated with the initiation of osteoarthritis.
1.2.3.2 Molecular mechanisms involved in cartilage degradation.

Often the different forms of arthritis are divided into inflammatory and non-inflammatory, where inflammatory indicates cellular inflammation resulting from the infiltration of various activated leukocytes into the joint and mediating cartilage destruction. Even though osteoarthritis has been usually referred to as a non-inflammatory pathology, due to the lack of infiltrating inflammatory cells in early stages of the disease, there is mounting body of evidence indicating that cartilage degradation is largely the result of chondrocyte-borne inflammation at a molecular level (Goldring, 2000b, Pelletier et al., 2001b, Attur et al., 2002). Abnormal mechanical forces appear to ‘awaken’ adult chondrocytes from a state of low metabolic activity and stimulate the production of a host of pro-inflammatory mediators. These include pro-inflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-8, IL-17, IL-18, monocyte chemoattractant protein 1 (MCP1) and reactive oxygen species (ROS; such as superoxide, hydrogen peroxide [H₂O₂] and peroxynitrite). These together with lipid-derived inflammatory stimuli (including prostaglandins and leukotrienes) serve to increase the catabolic activity of chondrocytes (Shlopov et al., 2000, Hardy et al., 2002, Fernandes et al., 2002, Gosset et al., 2010). The result is an increase in the release of matrix metalloproteinases (MMPs) and aggreganases, which can directly degrade the cartilage matrix. It has been proposed that, at least in part, the damage of the cartilage matrix might be an attempt of the chondrocytes to remove and replace the damaged matrix (Shlopov et al., 2000, Hardy et al., 2002, Fernandes et al., 2002, Gosset et al., 2010).

1.2.3.2.1 Cytokines.

In addition to age-related and biomechanical changes in the function of chondrocytes, inflammation, and the following deregulation of cytokine synthesis are major contributors to the misbalanced rate of anabolism and catabolism (Fernandes et al., 2002, Goldring and Berenbaum, 2004). Recently, in vivo and in vitro studies have demonstrated the involvement and the effects of pro-inflammatory cytokines, and particularly IL-1β and TNF-α, in the destruction of articular cartilage during osteoarthritis (Fraser et al., 2003, Goldring and Berenbaum, 2004, Kobayashi et al., 2005).
IL-1β, also known as catabolin, is a small, 17 kDa, cytokine protein encoded by the \( IL1B \) gene in humans (Auron et al., 1985, March et al., 1985). IL-1β, a member of the interleukin 1 cytokine family, is proteolytically activated by caspase 1 and has important functions in the inflammatory response. It is involved in various cellular activities, such as cell proliferation, differentiation and apoptosis (Oppenheim et al., 1989, Friedlander et al., 1996, Beasley and Cooper, 1999).

TNF-α, or cachexin, is a cytokine implicated in systemic inflammation, with particular relevance to acute phase reaction stimulation. It is a 17kDa protein, encoded by \( TNFA \) gene, with a primary role in the regulation of immune cells as a potent inducer of inflammation and apoptosis. Two receptors that recognize TNF-α have been identified and termed TNFR1 (TNF receptor type 1; p55/60) and TNFR2 (TNF receptor 2; p75/80), with TNFR1 being expressed by most tissues and TNFR2 being mainly expressed on the cells of the immune system (Locksley et al., 2001, Hehlgans and Pfeffer, 2005). Interestingly, the density of TNFR1 has been shown to be significantly greater in chondrocytes isolated from OA lesions compared to those isolated from areas not affected by the disease (Westacott et al., 1994), thereby proposing an important role for this cytokine in the progression of OA.

Upon binding to TNFR1, TNF-α, causes a conformation change in the receptors, which leads to the activation of the death domain (DD), enabling the adaptor protein (TNF-α receptor type 1 – associated death domain; TRADD) to bind to DD, and thus initiating a series of phosphorylation events. One group of proteins that mediate these phosphorylation events is the mitogen-activated protein kinase group, or MAPK-group (Garrington and Johnson, 1999), which is a serine-threonine kinase family that consists of the c-Jun N-terminal kinases (ERKs) and the p38 MAPK-activated protein kinases, downstream of pro-inflammatory cytokines, osmotic stress, or apoptotic signals (Lin et al., 1995, Aizawa et al., 2001). The stimuli first activate MAPK kinase kinases or MAPKKKs, which in turn phosphorylate and activate the MAPKK, kinase that then activates MAPK. MAPKK and MAPK then translocate into the nucleus of the respective cell, where they phosphorylate important transcription factors, including activating protein 1.
(AP-1; a member of the c-Jun family) which is especially involved in MMP13 transcription activation (Lim and Kim, 2011).

TNF-α and IL-1β are strong activators of the NF-κB signalling pathway in chondrocytes. First, TRADD recruits TNF-α receptor associated factor-2 (TRAF2) and RIP (receptor interacting serine-threonine protein kinase). TRAF2 in turn recruits the multi-component protein kinase IKK, enabling RIP-induced activation of it. The inhibitory IκBα is phosphorylated by IKK, which causes its subsequent degradation and ultimately the release and translocation of NF-κB into the nucleus, thereby activating a vast array of pro-inflammatory and catabolic genes (Liu-Bryan and Terkeltaub, 2010). However, it remains controversial whether inflammatory cytokines are primary or secondary regulators of articular cartilage degradation in osteoarthritis. Regardless, it has been shown that physiological loading of cartilage may protect against cartilage degradation by inhibiting the degradation of IKKβ activity in the canonical NF-κB transcriptional activation (Dossumbekova et al., 2007).

TNF-α and IL-1β together with a large number of other cytokines and growth factors trans-activate MMP promoters by convergence of AP-1 and ETS (E twenty six) through activation of p38, JNK and PKC signalling (Figure 1.3) (Iwamoto et al., 1990, Ahmed et al., 2003, Tower et al., 2003, Muddasani et al., 2007, Im et al., 2007, Sampieri et al., 2008, Lim and Kim, 2011). The induction of MMP13 promoter activity by IL-1β in chondrocytes has been shown to require one or more of the ETS sites and interaction between factors such as RUNX2 and AP-1 (cFos/cJun), which subsequently interact directly with the MMP13 promoter (Mengshol et al., 2001; Selvamurugan et al., 2004). Interestingly, PGE₂, an activator of both AP-1 and Fos-dependent promoters via PKA and PKC signalling pathways has been shown to dose-dependently inhibit the expression of MMP13, thereby suggesting a crosstalk between signalling pathways with adjacent transcriptional control elements and opposing effects on MMP13 transcriptional control (Li et al., 2004)
Figure 1.3 IL-1β and TNF-α molecular mechanism of action.

IL1R binds IL-1β but requires the IL1R accessory protein (IL-1RAcP) to transduce a signal. IL-1β binding causes activation of two kinases, IRAK-1 and IRAK-4, associated with the IL1R complex. IRAK-1 (IL1R Associated Kinase) activates and recruits TRAF6 to the IL1R complex. TRAF6 activates two pathways, one leading to NF-κB activation and another leading to c-jun activation. The TRAF associated protein ECSIT leads to c-Jun activation through the MAPK/JNK signalling system. TRAF6 also signals through the TAB1/TAK1 kinases to activate NIK, which phosphorylates IKK therefore triggers the degradation of IκB, and activation of NF-κB (Baud and Karin, 1999). TRAF2 (has been implicated in the activation of two distinct pathways that leads to the activation of Activation Protein-1 (AP-1) via the JNK, MEKK, p38 and together with RIP, NF-κB activation via the NIK (NF-κB Inducing Kinase). TNF-α activates MAPKs: ERK1 and ERK2 (Baud et al., 1999)
Other cytokines gaining increasing attention include IL-6, a potent pleiotropic cytokine, expressed by human chondrocytes in vitro and in vivo (Bender et al., 1990, Wang et al., 2010a,b), which has been suggested to play an important role in pathogenesis of osteoarthritis. It is thought to elicit both pro-inflammatory and anti-inflammatory effects in cartilage (Bender et al., 1990, Goldring, 2000a) through binding to IL-6 receptor (IL-6R), a two-subunit type I cytokine receptor. The IL-6 receptor alpha subunit (IL6Rα) contains the ligand-binding domain and IL-6 signal transducer component (gp130, also called CD130), which is shared by other cytokines from the IL-6 family (IL-11, IL-27, ciliary neurotropic factor [CNTF], cardiotropin [CT-1], CT-like cytokine [CLC], leukaemia inhibitory factor [LIF], oncostatin M; (Kishimoto et al., 1995). Binding of IL-6 to its receptor initiates a signal transduction cascade that activates JAK1/STAT3 (Signal Transducer and Activator of Transcription) and extracellular signal-regulated kinases (ERK)-1/2 pathways (Heinrich et al., 1998, Heinrich et al., 2003, Fischer and Hilfiker-Kleiner, 2007). The activated JAK1 phosphorylates STAT3, which dimerises and translocates into the nucleus, where it activates the transcription of STAT3 response elements (Hirano et al., 2000). IL-6 can be directly induced by TNF-α and IL-1β, although IL-6 on its own has been shown to directly augment the expression of MMP1 and MMP13 (Shlopov et al., 2000).

CXCL8, or IL-8 is a leucocyte chemotactic activating cytokine (chemokine), a member of the CXC chemokine family, encoded by the IL8 gene, with a primary function in angiogenesis, chemoattraction and activation of neutrophils (Modi et al., 1990, Harada et al., 1994a, Belperio et al., 2000). Various cell types produce IL-8 upon stimulation, as it is one of the major mediators of the inflammatory response (Akahoshi et al., 1994, Harada et al., 1994 a,b).

Chondrocytes have not only been found to express IL8, but the TNF-α-induced activation of NF-κB in chondrocytes also contributes to increased IL-8 protein production (Facchini et al., 2005). In fact, chondrocytes are a rich source of IL-8 and its synthesis is up-regulated in arthritic pathologies and by increased inflammatory cytokines levels (Borzi et al., 1999, Pulsatelli et al., 1999). The role of chemokines as second step mediators of local inflammation has gained increasing evidence (Baggiolini, 1998, Luster, 1998), whereby chondrocytes release IL-8 upon stimulation with IL-1β, TNF-α and LPS (Van Damme et al.,
Mian and colleagues have demonstrated that IL-8 is involved in NF-κB activation and the over-expression of MMP2 and MMP9 and that targeting of this cytokine with anti-IL-8 antibody leads to down-regulation of these proteases (Mian et al., 2003). That suggests that IL-8 can promote increase in matrix metalloproteinases expression, angiogenesis and neutrophil-mediated inflammation in the affected joint (Lotz et al., 1992, Strieter et al., 1992, Hu et al., 1993, Norrby, 1996), leading to further cartilage destruction, and makes it an interesting target for drug intervention.

1.2.3.2.2 Nitric oxide and Cyclooxygenase.

Among other inflammatory mediators found to be involved in the pathological process of OA is nitric oxide (NO). It has been shown that osteoarthritic cartilage secretes large quantities of NO and ROS in addition to high levels of nitrite/nitrates, detected in serum and synovial fluid of arthritis patients (Karan et al., 2003). This process might be caused by the increased expression of inducible nitric oxide synthase, or iNOS (Martel-Pelletier and Pelletier, 2010).

Two iso-forms of cyclooxygenase (COX), COX-1 and COX-2, catalyse the rate-limiting step in the biochemical conversion of arachidonic acid to prostaglandins and thromboxane A2 (TXA2) (Smith et al., 1996). COX-1 is a constitutively expressed protein that is thought to produce basal concentrations of prostaglandins and TxA2 necessary for normal physiologic functions in many tissues. Moreover, COX-dependent production of prostaglandin E2 (PGE2) occurs in many tissues through closely regulated and synchronized activities of cytoplasmic phospholipase A2 (cPLA2), COX, and PGE synthase (PGES, for review, see refs: (Murakami et al., 2000). COX enzymes catalyze the formation of prostaglandin H2 (PGH2) from arachidonic acid, followed by the isomerisation of PGH2 to PGE2 by PGES. COX-2, PGES, and their major pro-inflammatory product, PGE2, have been shown to be induced by pro-inflammatory cytokines (Murakami et al., 2000, Vane and Botting, 1998a).

Aberrant expression of COX-2 protein in articular cartilage concurs with significant increase in the levels of PGE2, an indication of osteoarthritis (Amin et al., 1997), leading to increased chondrocyte apoptosis (Notoya et al., 2000,
Pelletier et al., 2001a, Goldring and Berenbaum, 2004). The major prostaglandins synthesized by chondrocytes are PGE\(_2\) and PGD\(_2\). Although some studies have suggested that PGE\(_2\) has anabolic effects at low concentrations (DiBattista et al., 1996) and that it can suppress MMP13 and MMP1-mediated catabolism (Tchetina et al., 2007), higher concentrations of this prostaglandin, such as those reached during inflammation lead to severe cartilage erosion and chondrocyte apoptosis (Amin et al., 1997, Attur et al., 2008). Zhu and collages show that exogenously added PGD\(_2\) diminishes T/C-28a2 chondrocyte cell line viability (Zhu et al., 2010).

In addition to regulating metalloproteinases, pro-inflammatory cytokines such as IL-1\(\beta\) and TNF-\(\alpha\) have been shown to suppress the expression of COL2A1 by chondrocytes *in vitro* (Reginato et al., 1993, Goldring et al., 1994 a,b). In contrast, increased anabolic activity of chondrocytes has been observed in osteoarthritic cartilage, possibly due to the cytokine-induced PGE\(_2\) formation, which up-regulates COL2A1 transcription (Goldring et al., 1994b, Miyamoto et al., 2003).

### 1.2.3.2.3 Anti-inflammatory cytokines and proteins.

In addition to the above mentioned pro-inflammatory cytokines, human chondrocytes have been shown to produce both IL-10 as well as the IL-10 receptor (IL10R) with significantly higher levels in osteoarthritic chondrocytes compared to normal chondrocytes (Iannone et al., 2001). IL-10 is a pleiotropic cytokine with important immuno-regulatory functions, whose actions influence activities of many of the cell types in the immune system. IL-10 has potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-\(\alpha\), IL-6 and IL-1\(\beta\) by activated cells. IL-10 receptor (IL10R) is a complex of tetramers consisting of two ligand-binding subunits (IL10R1) and two accessory signalling subunits (IL10R2). Upon binding of IL-10 to the extracellular domain of IL10R1, JAK1 and Tyrosine Kinase-2 (TYK2), which are constitutively associated with both IL10R1 and IL10R2, respectively, are activated by phosphorylation (Riley et al., 1999). These kinases then phosphorylate specific tyrosine residues on the intracellular domain of IL10R1, which when phosphorylated, serve as temporary docking sites for STAT3. STAT3 is then
phosphorylated by JAK1 and translocates to the nucleus, where it binds with high affinity to SBE (STAT-Binding Elements) in the promoters of various IL-10 responsive genes (Riley et al., 1999). IL-10 has also been reported to interfere with the activation of p38/MAPK pathway, thus inhibiting the activation of NF-κB. Direct stimulation of OA chondrocytes with IL-10 has been shown to inhibit the TNF-α-dependent activation of osteoarthritic chondrocytes and therefore to down-regulate the expression of MMP1 and MMP13 (Shlopov et al., 2000). Interestingly, IL-10 (Lee and Chau, 2002) induces heme oxygenase-1 (HO-1), which is expressed and functionally active in chondrocytes from OA cartilage (Fernandez et al., 2003). HO-1 is implicated in the protection against tissue injury and is modulated by cytokines such as IL-1β and TNF-α, the latter of which down-regulate HO-1(Fernandez et al., 2003).

HO-1 expression can be strongly induced at transcription level in most tissues by various stress factors including heavy metal, heat shock, ultra violet radiation, endotoxin, hypoxia, hyperoxia, ischemia, hydrogen peroxide, pro-inflammatory cytokines (i.e. IL-1β, TNF-α), NO, cellular glutathione storage depletion and high levels of its substrate heme (Wagener et al., 2003). Contained within the promoter region of HO-1 are the oxidative stress-responsive transcription factor NF-E2-related factor 2 (Nrf2), NF-κB, AP-1, AP-2 and CREB. Alternatively, HO-1 can be negatively regulated by agents such as scavengers of ROS due to their ability to reduce oxidative stress within the body (Lautier et al., 1992). Primarily, HO-1 induction prevents cellular damage and tissue injury by removing excess free heme within the body. In addition, heme acts as a prosthetic group for the activity of inflammatory enzymes including iNOS and COX-2 (Willis, 1999) and is involved in the generation of ROS and RNS that causes cellular stress (Jeney et al., 2002).

1.2.3.2.4 Matrix metalloproteinases in OA.

Proteinases are involved in essential steps of cartilage and bone homeostasis. Normal cartilage ECM is in a state of dynamic equilibrium, with a balance between anabolism and catabolism, brought about by the balance between the proteinases that degrade the cartilage and their inhibitors. It is believed that a disturbance of this balance in favour of catabolism, leads to the pathological
degradation of cartilage ECM observed in osteoarthritis (Kevorkian et al., 2004). MMPs are induced by TNF-α, IL-1β, which in combination with IL-6, IL-8, oncostatin M, and other pro-inflammatory mediators, synergistically enhance MMPs production in vitro, ex vivo and in vivo (Shlopov et al., 2000, Hui et al., 2003, Hall et al., 2003, Cawston et al., 2003).

MMPs are responsible for the enzymatic cleavage of peptide bonds (Turk, 2006, Lah et al., 2006) for the completion of many biological processes, such as digestion, blood coagulation, immune functions, development, apoptosis and processing of precursors needed for collagen synthesis (Dickinson, 2002). Proteases selectively hydrolyse a peptide bond in the polypeptide chains of the target protein and are subdivided into two main groups depending on the location of the preferred peptide bond – endopeptidases and exopeptidases. Exopeptidases are subdivided into aminopeptidases or carboxypeptidases – peptidases that cleave substrate molecules at the amino-terminal or the carboxy-terminal position, respectively (Barrett, 1980, Cawston and Wilson, 2006). Endopeptidases cleave in the middle of the target polypeptide chain and can be subdivided into groups depending on the chemical group involved in the process of catalysis – aspartic-, threonine- and cysteine-proteinases (intracellular, functioning at acid pH), or serine- and metallo-proteinases (extracellular, act at neutral pH; Figure 1.4)(Cawston and Wilson, 2006).

Degradation of the components of ECM is accomplished by a family of more than 26 zinc-containing endopeptidases termed matrix metalloproteinases (MMPs), which are either secreted into the extracellular space, or attached to the plasma membrane of the respective cell (Visse and Nagase, 2003). They share homologous amino acid sequences, contain conserved domains related to their specific substrate preference, and allow recognition of other proteins (Visse and Nagase, 2003). As a family, MMPs can degrade nearly all of the cartilage ECM components, although each individual member is specialized in cleaving specific molecules.
In general, MMPs are composed of three distinct domains (Rannou et al., 2006): a pre-domain, needed for enzyme maturation and secretion from the cell; pro-domain, required for the maintenance of the enzyme in inactive state; and the catalytic domain, which contains a zinc atom and performs the hydrolysis of the peptide bond. MMPs are categorized into at least five main groups, according to their substrates, cellular localization and primary structure: collagenases, gelatinases, stromelysins and membrane bound (MT)-MMPs (Table 1.1; Figure 1.4). MMPs are tightly controlled at several levels – transcriptional control, pro-enzyme activation, and inhibition of active enzymes by endogenous inhibitors (Chakraborti et al., 2003). At transcriptional level, MMPs gene expression is regulated by pro-inflammatory cytokines and growth factors. After binding to their specific receptors, these stimuli activate an intracellular cascade of events, leading to the activation of AP-1 transcription factors and that leads to the up-regulation of MMPs expression (Martel-Pelletier et al., 2001).
### Table 1.1 Groups of MMPs.

<table>
<thead>
<tr>
<th>Group</th>
<th>MMPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td>MMP1 (collagenase-1)<em>, MMP8 (collagenase-2), MMP13 (collagenase-3)</em>, MMP18 (collagenase-4)</td>
</tr>
<tr>
<td>Gelatinases</td>
<td>MMP2 (gelatinase A, MMP9 (gelatinase B)</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>MMP3 (stromelysin-1)*, MMP10 (stromelysin-2), MMP11 (stromelysin-3)</td>
</tr>
<tr>
<td>Matrilysins</td>
<td>MMP7, MMP26</td>
</tr>
<tr>
<td>MT-MMPs</td>
<td>MMP14 (MT1-MMP), MMP15 (MT2-MMP), MMP16 (MT3-MMP), MMP17 (MT4-MMP), MMP24 (MT5-MMP), MMP25 (MT6-MMP)</td>
</tr>
</tbody>
</table>

MMPs, matrix metalloproteinases; MT, membrane type.

*Proteases studied in the present study.

Another mechanism of regulation is the secretion of pro-enzymes MMPs. The proteinases are secreted in inactive form, and require the removal of the pro-domain by MT1-, MT2-, MT5-MMP, plasmin, uPA (urokinase) for activation (Chakraborti et al., 2003). Upon removal of the pro-domain, MMPs become transiently unstable and undergo conformational change, involving a dissociation of a cysteine residue from the zinc atom in the catalytic domain, thereby exposing the active site and allowing for trans-auto activation of the MMP (Chakraborti et al., 2003).

Most cells in the body express MMPs, even though some proteases are associated with a particular cell type. For example the principle substrate for MMP2, or gelatinase A and MMP9, or gelatinase B is the type IV collagen. MMP3, also called stromelysin activates MMP1, or collagenase-1, and is able to cleave a broad range of matrix proteins (Vincenti et al., 1996). Both MMP1 and MMP3 are amongst the most ubiquitously expressed collagenases, unlike
MMP13, or collagenase-3, which is expressed primarily by cartilage and bone during development and by the chondrocytes in OA (Borden and Heller, 1997, Mengshol et al., 2000, Martel-Pelletier et al., 2001, Kevorkian et al., 2004).

The only mammalian interstitial proteases that can specifically cut triple-helical interstitial collagens (type and type II) at natural pH are collagenases (Gadher et al., 1990, Kevorkian et al., 2004), which cleave at a single locus three quarters from the N-terminus, leading to the unwinding of the α-chains. The major collagenases able to perform this enzymatic reaction (MMP1, MMP8 and MMP13) were found elevated in OA cartilage compared to normal tissue (Reboul et al., 1996; Martel-Pelletier et al., 2000). The specific role of MMP8, however, in OA progression remains to be investigated.

IL-1β and TNF-α are thought to be the most important cytokines relevant to osteoarthritis, with increased levels in OA chondrocytes (Tetlow et al., 2001). They are thought to down-regulate anabolic genes such as aggrecan and type II collagen, as well as to directly up-regulate the expression of MMP1, MMP3 and MMP13 (Shlopov et al., 1997, Bau et al., 2002). MMP13 specifically hydrolyses type II collagen more rapidly and efficiently than MMP1 and MMP8 (Mitchell et al., 1996). Chondrocytes obtained from cartilage adjacent to OA lesions expressed high levels of both MMP1 and MMP13 (Shlopov et al., 1997).

Kevorkian and colleagues conducted the expression profiling of MMPs in cartilage samples obtained from the hips and knees of normal and OA patients (Kevorkian et al., 2004). Examination of collagenases showed that MMP8 expression was undetected thus suggesting that it is unlikely to play a role in cartilage destruction. MMP9 and MMP2, however, are significantly over-expressed in OA cartilage. The inducible MMP9 is thought to have a secondary role, since it contributes to cleaving collagen fibres only after the chains of the triple helix have been already cleaved by the interstitial collagenases (Stetler-Stevenson et al., 1997). In contrast MMP2 and MMP14 (MT1-MMP) are both constitutively expressed and minimally regulated.

MMP13 expression is highly increased in OA, consistent with the belief that it is the predominant protease in osteoarthritis (Kevorkian et al., 2004). Interestingly, MMP1 expression decreased in cartilage samples from patients with hip OA, but
was found to be highly increased in osteoarthritic knee cartilage, suggesting that the regulation of this collagenase may differ between joints. A dramatic decrease in \textit{MMP3} in diseased knee cartilage has also been found, and therefore a maintenance function of this protease, which is dysregulated in OA, is suggested (Kevorkian \textit{et al.}, 2004; Bau \textit{et al.}, 2002).

\textbf{1.2.3.2.5 Chondrocyte apoptosis and OA.}

Apoptosis, or programmed cell death, is a normal process during which an orchestrated sequence of events leads to cell death, primarily executed by enzymes called caspases (Kerr \textit{et al.}, 1972). Apoptosis is critical not only during development and tissue homeostasis, but also in the pathogenesis of a variety of diseases. Recent studies have revealed that apoptosis is associated with the onset and development of OA (Blanco \textit{et al.}, 1998, Kim \textit{et al.}, 2000, Sharif \textit{et al.}, 2004, Musumeci \textit{et al.}, 2011), whereby the proportion of apoptotic cells in OA is greater compared to that in healthy cartilage (Aigner \textit{et al.}, 2001). The expression of several caspases, such as caspase-3 and caspase-8, has been found to be increased in human osteoarthritic cartilage and in animal models of the disease (Sharif \textit{et al.}, 2004, Robertson \textit{et al.}, 2006)

Caspases are a unique family of cysteine-aspartic proteases (Alnemri \textit{et al.}, 1996), which are triggered in early stages of apoptosis and are responsible for the activation of most of the events leading to cell death, by cleaving more than a dozen protein kinases, including focal adhesion kinases (FAK), Protein Kinases B & C (PKB & PKC), Raf1 and Lamins. The latter make up the inner lining of the nucleus, and their destruction leads to disintegration of the nuclear lamina, and shrinkage of the nucleus (Kerr \textit{et al.}, 1972)

Apoptosis can be triggered by intrinsic stimuli such as DNA damage, or by extrinsic stimuli such as cytokines. One of the major cytokines, produced by variety of cells in response to infection or multitude of inflammatory stimulants is TNF-\(\alpha\). Activation of its receptor, TNFR1, a member of a family of death receptors is involved in the initiation of apoptosis. It is suggested that the TNFR1 is a preassembled trimer, located on the plasma membrane, the cytoplasmic domain of each subunit of which contains a segment of about 70 amino acids called “death domain” (DD). Death domains of TNF-\(\alpha\) receptor specifically self-
associate to induce cell death, by either activating AP-1 and NF-κB (Figure 1.5), or inducing apoptosis. Binding of TNF-α to its receptor causes a conformational change in the death domain, which initiates the recruitment of a number of proteins as indicated in Figure 1.5, two pro-caspases join the protein complex and undergo proteolytic cleavage that yields active caspase-8. Caspase-8 is called an initiator caspase, because it instigates the apoptotic process by activating effector caspases, such as caspase-3, directly involved in carrying out apoptosis (Le et al., 2002).
Activated TNFR1 leads to the recruitment, oligomerization, and activation of Domain) and RIP (Recepto

Figure 1.5 Molecular mechanism of caspase-driven programmed cell death. Initially, TRADD (TNFR-Associated Death Domain) protein, binds to TNFR1. Then, TRADD recruits FADD (Fas-Associated Death Domain), RAIDD (RIP-Associated ICH-1/CED-3-homologous protein with a Death Domain), MADD (MAPK Activating Death Domain) and RIP (Receptor-Interacting Protein). Binding of TRADD and FADD to TNFR1 leads to the recruitment, oligomerization, and activation of Caspase-8. Activated Caspase-8 subsequently initiates a proteolytic cascade that includes effector Caspases (Caspases 3, 6, 7) and ultimately induces apoptosis.
1.2.4 Current therapies for the treatment of OA.

A vast array of novel therapies have been developed over the last decade or so for the treatment of arthritic pathologies, especially RA, which now sit alongside the more traditional approaches that include methotrexate, non-steroidal anti-inflammatory drugs (NSAIDs), and glucocorticoids (for review, see: Getting et al., 2009). The most widely used therapies for OA are physiotherapy; non-selective COX-1/2 inhibitors such as ibuprofen, naproxen, usually prescribed with protein pump inhibitors such as omeprazole, to alleviate GI side-effects; COX-2 selective inhibitors, or ‘coxibs’ are another choice of therapy, usually given with low dose aspirin, to prevent CV side-effects, and a proton pump inhibitor. A third choice of therapy is glucocorticoids, prednisone being the drug of choice, administered via intra-articular (i. a.) injection.

1.2.4.1 Non-steroidal anti-inflammatory drugs (NSAIDs).

NSAIDs form an important class of drugs, the therapeutic applications of which have spanned several centuries. The ability of NSAIDs to treat fever and inflammation dates back about 3500 years ago to the time when Hippocrates prescribed an extract from willow bark. In the 17th century, the active ingredient of the bark salicin was discovered, in 1860, the German Kolbe company started producing salicylic acid for the first time and in 1899, Bayer started mass production of Acetylsalicylic acid, or as we know it aspirin (Vane, 2000). The mechanism of action of drugs like aspirin and Indomethacin was revealed by Professor Sir John Vane ~ 40 years ago (Vane, 1971).

The primary effect of NSAIDs is pain relief, but they also exhibit anti-pyretic and anti-inflammatory effects (Vane, 1971) but do not modify the underlying causes of chronic diseases, and these drugs are thus prescribed for symptomatic relief only. The success of the NSAIDs in treating inflammatory disorders such as RA and OA was due to its inhibitory effects on the enzyme cyclooxygenase (COX) (Vane and Botting, 1998b) NSAIDs inhibit prostaglandin synthesis, which can lead to gastrointestinal (GI) toxicity, limiting its use in chronic inflammatory conditions such as OA (Tamblyn et al., 1997). Seminal work by Prof. Sir John Vane, Prof. Roderick J. Flower and colleagues led to the identification of two iso-
forms of COX enzyme – COX-1 and COX-2 (Flower and Vane, 1972). The traditional NSAIDs, prescribed for the treatment of joint pain and inflammation, are known to bring about their anti-inflammatory effects by non-selective inhibition of COX activity. However, during inflammation, expression of COX-1 levels do not change, whilst COX-2 levels are significantly up-regulated (Rao and Knaus, 2008), leading to increased production of pro-inflammatory prostaglandins. COX-1 on the other hand does not appear to be associated with inflammation, but is highly expressed in the GI tract (Kargman et al., 1996), where it is involved in the production of cytoprotective 

\[
PGE_2 \quad \text{and} \quad PGI_2
\]

thereby reducing gastric acid secretion (by the parietal cells in the stomach), up-regulating mucosal blood flow and initiating production of viscous protective mucus. This explains the adverse effect of the traditional NSAIDS (Lazzaroni and Bianchi Porro, 2004). COX-1 isoform is also widely expressed in platelets, where they are responsible for the formation of pro-aggregatory thromboxane A2 (TxA2).

These adverse effects led to the development of selective COX-2 inhibitors to bring about a more targeted and specific anti-inflammatory effect (Lazzaroni and Bianchi Porro, 2004). However, post-marketing pharmacovigilance of selective COX-2 inhibitors including celsecoxib and rofecoxib (VIOXX), highlighted an ~5 fold increase in myocardial infarction (Mukherjee et al., 2001), explained by the effect of the selective COX-2 inhibitors on PGI2 production. PGI2 is a vasodilator and a potent inhibitor of platelet aggregation (McAdam et al., 1999), which was significantly attenuated by the selective COX-2 inhibitors. Although selective COX-2 inhibition has no effect on TxA2 production, they tip the natural balance between the TxA2 and PGI2 production, thus leading to an increased risk of thrombotic cardiovascular event as demonstrated more recently (Cannon et al., 2006). Studies showed that selective inhibition, knock down or mutagenesis of COX-2, or deletion of the receptor for PGI2, accelerated thrombogenesis and elevated blood pressure in mice, an effect found to be attenuated by COX-1 knock-down (Cheng et al., 2006). Discoveries like this led to the withdrawal of the very selective COX-2 inhibitor rofecoxib from the market in September 2004, just 5 years after it was introduced.
1.2.4.2 Glucocorticoids.

Glucocorticoids are endogenously produced hormones implicated in carbohydrate and protein metabolism and exert a potent regulatory effect on inflammation, innate and acquired immune response (Auphan et al., 1995). Additionally, they are potent anti-inflammatory drugs inhibiting the synthesis of pro-inflammatory cytokines, interleukins and iNOS, and the production of PGE$_2$ by inhibiting the transcription of COX-2.

Glucocorticoid receptor is a member of the family of nuclear receptors, which upon binding to their cognate ligands, migrate to the nucleus and act as positive or negative gene transcription regulators (Schacke et al., 2004)(Figure 1.6). In the case of positive regulation they trans-activate glucocorticoid response elements (GREs) that are located on the promoter region of the responsive genes (Beato et al., 1995, Mangelsdorf et al., 1995, Kastner et al., 1995). Glucocorticoid receptors are able to negatively regulate transcriptional activation of certain genes in two descriptive mechanisms, one of which is referred to as trans-repression. Trans-repression involves repression of transcription factor AP-1, thus inhibiting its dissociation from the promoter (Konig et al., 1992). Another mechanism of action of glucocorticoid receptors is the repression of the NF-$\kappa$B transcription factor family by physically associating with p65 subunit (Mukaida et al., 1994, Ray and Prefontaine, 1994, Caldenhoven et al., 1995) In addition, in most cell types excluding endothelial cells, the glucocorticoid receptors trans-activates the I$\kappa$B$\alpha$ gene transcription, thus leading to prevention of NF-$\kappa$B translocation in the nucleus (Auphan et al., 1995, Scheinman et al., 1995, Brostjan et al., 1996).

Glucocorticoids are effective anti-inflammatory therapies; however side effects, including suppression of the immune response, healing impairment and gastric ulceration, can limit their use (Schacke et al., 2002). When used as anti-inflammatory agents, glucocorticoids also influence water and electrolyte balance, leading to Cushing’s syndrome, easy bruising, and redistribution of the fat in the abdominal region. Additionally, glucocorticoid drugs influence bone density by Ca$^{2+}$ regulation and phosphate metabolism (Guaydier-Souquieres et al., 1996). While these adverse effects are not a problem in short-term oral therapy, or more localized application of the drugs (such as i.a. injections or topical ointments), they severely limit the long-term use of glucocorticoids in chronic diseases such as RA and OA.
Figure 1.6 Glucocorticoids mechanism of action.

Glucocorticoid receptors are maintained in an inactive oligomeric complex by regulatory proteins such as HSP90 (Heat Shock Protein 90 kD), which after dimerizing bind to HSP70, FKBP52 and p23 phosphoprotein, all of which form the GR inhibitory complex. Upon binding to its glucocorticoid ligand in the cytoplasm, the inhibitory complex dissociates, induces conformational change that exposes the nuclear localization signal of the GR, which then translocates to the nucleus (Pelaia et al., 2003). There, the GR negatively regulate (trans-repress) gene transcription via physical interactions with transcription factors, activated by various pro-inflammatory stimuli (cytokines, etc.) such as the components of AP-1 – c-Jun and c-Fos, and the p65 domain of NF-κB (Schoneveld et al., 2004, Davies et al., 2005). Other mechanisms by which glucocorticoids NF-κB-mediated activation of pro-inflammatory genes are by reducing the levels of phosphorylation of the carboxy-terminal domain of RNA Polymerase II, which is essential for the recruitment of this enzyme to the promoter region and by increasing the transcription and synthesis of IκB, thus causing NF-κB retention into the cytoplasm. The glucocorticoid family are also suggested to negatively regulate the MAPK family (JNK, ERK1, ERK2 and p38), therefore exerting their anti-inflammatory effects (Li et al., 2003).
1.2.4.3 Biologicals.

TNF-α is a primary pro-inflammatory cytokine detected in the synovium and plasma of RA patients and OA cartilage, and is known to be able to activate an array of pro-inflammatory cytokines, including IL-1β, IL-6, IL-8 and MCP-1. Anti-inflammatory therapy with monoclonal antibodies (mAbs) directed against TNF-α has emerged in the 1990’s as a major advancement in the treatment of various immune-mediated diseases such as RA, ankylosing spondylitis, psoriatic arthritis and Crohn's disease. A huge boost to anti-inflammatory therapy developments has been the launch of the protein-based injectable etanercept (Enbrel®), and anti-TNF antibodies infliximab (Remicade®), adalimumab (Humira®) (Palladino et al., 2003, Wong et al., 2008), also referred to as ‘biologicals’. These anti-TNF monoclonal antibodies exert their beneficial effects by scavenging the pro-inflammatory cytokine TNF-α.

Infliximab is a chimeric monoclonal anti-TNF-α antibody, which binds to soluble and membrane-bound TNF-α, but not to TNF-β, and is able to effectively regulate and mediate inflammatory processes involved in a number of different inflammatory diseases such as RA, Crohns disease, and ulcerative colitis (Bingham, 2008, Haveran et al., 2011). Other anti-cytokine drugs available on the market are etanercept – a TNF receptor covalently linked to the Fc chain of IgG molecule, and anakinra – an IL-1 antagonist. Infliximab, adalimumab and etanercept inhibit the effect of TNF-α by binding to it, although etanercept can additionally bind to lymphotoxin-α. This can be of interest in the treatment of juvenile arthritis, due to lymphotoxin-α production by inflamed tissues (Heiligenhaus et al., 2011, Sevcic et al., 2011).

Even though a beneficial effect has been assigned to these drugs in the clinical management of chronic arthritis and other inflammatory diseases, biologicals lead to a marked increase in opportunistic infections including tuberculosis (TB) with a 3-4-fold higher risk in patients taking infliximab compared to etanercept (Dixon et al., 2010). In part, this could be due to latent TB and so routine screening of individuals prior to commencing therapy is necessary. A recent review by Wallis identified that TNF antibodies pose a greater risk than soluble TNF-α receptor antagonists (Wallis, 2009). A good example is Infliximab, which
when used for prolonged period of time to treat inflammatory disease such as inflammatory bowel disease and Crohn disease causes psoriasis, urinary tract infections, nausea, rashes, decreased white and red cell count and platelet count, vasculitis, and development of lymphoma (Lee et al., 2007, Ko et al., 2009, Fiorino et al., 2009). A number of cytokine antagonists are in development for treatment of RA, including those that target IL-6 (tocilizumab; approved for use in Japan), IL-15, and RANKL, as well as kinase inhibitors, including those that inhibit JAK-3 and SyK (Bingham, 2008). Although some patients respond well to these agents, development of novel therapies is essential should patients become refractory to current therapies.

1.2.4.4 Endogenous novel therapeutics

Annexin 1 is a member of the annexin superfamily of calcium and phospholipid binding proteins (Gerke and Moss, 2002, Perretti and Gavins, 2003). Due to its up-regulation by glucocorticoids, Annexin 1 has been suggested to function as a cellular mediator of the anti-inflammatory effects of glucocorticoids (Roviezzo et al., 2002). Cytokines such as IL-1β, TNF-α and IL-6 have been shown to augment Annexin 1 expression (Perretti and Gavins, 2003). A variety of functions have been attributed to this peptide, including inhibition of cell proliferation (Croxtall and Flower, 1992), anti-migratory and anti-inflammatory effects (Flower and Rothwell, 1994, Philip et al., 1997, Gerke and Moss, 2002, Perretti et al., 2002), cell differentiation regulation, and membrane trafficking (Diakonova et al., 1997, Traverso et al., 1999). The anti-inflammatory properties of Annexin 1 have been already demonstrated in animal models of RA and myocardial infarction (Perretti et al., 1993, Yang et al., 1999, La et al., 2001)

The potential involvement of Annexin 1 in human conditions has been best described in RA, where the endogenous peptide inhibits experimental arthritis in animal models, and upon treatment with anti-annexin-1 antibody, the disease progression exacerbates severely and prevents glucocorticoid drug-mediated anti-inflammatory effects (e.g. dexamethasone) and up-regulates PGE₂ and TNF-α production by the synovium (Yang et al., 1997). These data suggested that advocating administration of annexin-1 derivatives, or non-glucocorticoid
manipulation of endogenous annexin-1 expression, may prove a beneficial treatment approach in arthritis and other inflammatory diseases.

In concordance with the increasing interest in investigating the properties of endogenous anti-inflammatory mediators, the family of galectins is progressively examined for their immuno-regulatory and anti-inflammatory properties. Galectins (type of lectins, which bind to β-galactosidase), of which 14 mammalian subtypes have been identified, are wildly distributed in animals and possess a variety of functions, including inhibition of chronic inflammation (Dumic et al., 2006, Norling et al., 2009).

Interest has been directed towards Galectin-1, -3 and -9, as these have been identified as important players in modulation of both acute and chronic inflammatory diseases (Norling et al., 2009). Galectin-1 exhibited anti-inflammatory and immunosuppressive properties in a range of models of inflammatory disorders including colitis (Santucci et al., 2003), arthritis (Rabinovich et al., 1999) and diabetes (Perone et al., 2006). Galectin-3 is found in various epithelial cells and cartilage, as well as inflammatory cells such as macrophages, suggesting a role in inflammation (Henderson and Sethi, 2009). Increased expression of Galectin-3 and -9 have been detected in synovium of rheumatoid arthritis patients and to a lesser extent in osteoarthritic synovium (Ohshima et al., 2003, Seki et al., 2007), whereas Galectin-1 expression is down-regulated in synovium from patients with juvenile idiopathic arthritis (Harjacek et al., 2001). Galectin-3 has been implicated in a variety of processes such as heart failure, fibrogenesis, tissue repair, inflammation, cancer and stroke (van Kimmenade et al., 2006, Yan et al., 2009a, Yan et al., 2009b, Liu et al., 2009, de Boer et al., 2009).

Another class of endogenously produced peptides, with anti-inflammatory and immunosuppressive efficiencies are the melanocortin peptides (including α-MSH) found to be efficacious in experimental models of allergic inflammation (Grabbe et al., 1996, Luger et al., 2000), chronic inflammation (Ceriani et al., 1994), systemic inflammation (Delgado Hernandez et al., 1999), inflammatory bowel disease, brain inflammation (Rajora et al., 1997a,b) and autoimmune uveoretinitis (Taylor and Namba, 2001). A mechanistic approach has been identified for α-MSH binding to a family of Rhodopsin-like seven-transmembrane
G-protein coupled receptors, whose activation leads to increase in cyclic adenosine monophosphate (cAMP), thereby preventing NF-κB activation via preservation of IκBα protein and resulting in the reduction of production and synthesis of pro-inflammatory cytokines (Catania et al., 2004).

1.3 Melanocortin system.

The “melanophore stimulants” were discovered about 90 years ago during surgical ablation experiments by Hogben and Winten (Hogben and Winton, 1922), in which the pituitary gland has been shown to be implicated in the change of skin colour of amphibian. This led to the recognition of pars intermedia as the origin of biological principle. In the 1950s an isolated frog skin assay was developed, which led to the extraction, molecular characterisation and sequence determination of melanocyte-stimulating hormones (MSHs; melanotropins) (Shizume et al., 1954).

Melanocortins are ancient peptides which can be traced back over 700 million years and are derived from the pro-opiomelanocortin (POMC) gene (Heinig et al., 1995). The melanocortin system has been shown to play numerous roles including melanogenesis, energy homeostasis regulation and modulation of inflammatory pathways, obesity, cardiovascular and sexual health (Wikberg et al., 2000). The discovery of anti-inflammatory and immuno-modulatory functions has led to a renewed interest in alpha-melanocyte stimulating hormone (α-MSH), which along with its related peptides and their cognate receptors are responsible for the transmission of these effects and might present potential treatment options for inflammatory diseases (Getting, 2002, Catania et al., 2004).

The isolation and sequence determination of adrenocorticotrophic hormone (ACTH), β-lipotropin and γ-lipotropin demonstrated that the sequence for α-MSH was formed following proteolytic cleavage of ACTH (Nakanishi et al., 1979). These findings led to the hypothesis that the longer peptides might be serving as a precursor for the shorter proteins (Nakanishi et al., 1979). In 1979 and after deducing the whole amino acid sequence of the POMC peptide from its cDNA, Nakanishi and colleagues reported that it contains an unknown MSH-like peptide sequence, and identified a region bearing homology with α-MSH and β-MSH within the N-terminal fragment in a core heptapeptide (Nakanishi et al., 1979).
Due to the region separation by a dibasic amino acid cleavage sites, they predicted that the intervening peptide will be a secretory product and termed it γ-MSH, due to the high homology to α-MSH and β-MSH. In the last 30 years POMC-derived molecules from many different species were analysed and their genes sequenced. It has been confirmed that POMC gene is expressed in many different tissues and the bioactive peptides, resulting from its post-translational processing, function as not only neuro-peptide regulators in the brain, but also in the periphery (D'Agostino and Diano, 2010).

1.3.1 Pro-opiomelanocortin (POMC) protein.

The POMC gene is 241 amino acids in humans (Takahashi et al., 1981), and 209 in mouse (Uhler and Herbert, 1983) and rat (Drouin and Goodman, 1980). POMC is actively transcribed in several tissues, including the hypothalamus, pituitary and periphery including the immune system, lungs, spleen, melanocytes and the gastrointestinal tract (Wikberg et al., 2000). POMC is functionally inert, but undergoes extensive and tissue-specific post-translational modification in order to generate the range of smaller and biologically active peptides mentioned earlier (Rafin-Sanson et al., 2003; Figure 1.7). POMC is comprised of three domains: the N-terminus region, which contains γ-MSH (melanocyte stimulating hormone); the central highly conserved ACTH_{1-39} sequence with α-MSH at its N-terminus; and the C-terminal β-lipotropin, which can be cleaved to generate β-endorphin (Castro and Morrison, 1997). Many hormones and neurotransmitters are synthesized as large pro-hormones, which require cleavage in order to release biologically active fragments. Within the ACTH_{1-13} sequence and the endorphin system, the cleavage sites are usually located between two pairs of basic amino acid residues (-KK-, -RK-, -RR-, -KR-). There are seven members of the pro-hormone convertases (PC) family including PC1/3, PC2, furin/PACE, PACE4, PC4, PC5/6, and PC7/SPC7/LPC/PC8 (von Eggelkraut-Gottanka and Beck-Sickinger, 2004) and the range of processing enzymes expressed in particular tissue, defines the repertoire of POMC-derived products. Thus, PC1 cleaves POMC into ACTH_{1-39} and β-lipotropin, together with low concentrations of β-endorphin, whilst PC2 cleaves POMC into β-endorphin and β-MSH (Figure 1.7), but not ACTH (Benjannet et al., 1991).
Figure 1.7 Bioactive peptides formed following the post-translational processing of POMC.

POMC in mammals is composed of 3 exons, of which exons 2 and 3 are translated into protein. Prohormone convertases 1 and 2 (PC1/2) break the parent POMC peptide into successively smaller peptides by cleavage at paired dibasic amino acid residues consisting of lysine (K) and/or arginine (R). Cleavage of pro-ACTH by PC1 gives rise to N-POC and joining peptide (JP). The final products are generated in a tissue specific manner by PC2, for example α-MSH and ACTH. They also involve additional enzymatic post-translational modifications, such as in the formation of mature α-MSH - removal of basic amino acid residues by the C-terminal by carboxypeptidases (CPE), amidation by α-amidating mono-oxygenase (PAM) and finally acetylation of α-MSH by N-acetyltransferase (N-AT). The final products include the melanocortins (MSHs and ACTH), β-endorphin and corticotrophin-like intermediate peptide (CLIP). There are intermediate peptides whose biological function remains unclear, such as β-lipotrophin and γ-lipotrophins. All melanocortin peptides contain the common amino acid motif sequence, with ACTH and α-MSH also having in common the tripeptide KPV, which has been shown to antagonize IL-1β effect (highlighted in red).
1.3.2 Melanocortin receptors: Binding and distribution.

Melanocortin peptides exert their biological effects by binding to the smallest family of membrane bound guanine nucleotide-binding protein-coupled receptors (GPCRs). These are composed of seven transmembrane spanning \( \alpha \)-helices connected by three intracellular and three extracellular loops and short extracellular N-terminus and short intracellular C-terminus (Catania et al., 2004). All melanocortin receptors possess two highly conserved cysteine residues in their C-terminus that form covalent disulphide bonds and therefore stabilize the receptor structure.

The melanocortin receptor (MC) family consists of five members that have been cloned and termed MC\(_1\) to MC\(_5\). Each receptor is positively coupled to adenylate cyclase; with activation leading to cAMP accumulation following ligation by the common, core tetrapeptide sequence His-Phe-Arg-Trp (HFRW; Wikberg et al., 2000; Getting, 2006). Even though \( \alpha \)-MSH has equal affinity to MC\(_3\) and MC\(_4\), it has been shown to be somewhat specific to MC\(_1\) (Schioth et al., 1996, 2005; Siegrist and Eberle, 1995). Conversely, \( \beta \)-MSH, \( \gamma \_3 \)-MSH and ACTH have been shown to have the highest affinity for MC\(_4\) (Schioth et al., 1996, Harrold et al., 2003), MC\(_3\) (Gantz et al., 1993a) and MC\(_2\) (Getting, 2006) respectively.

MCs are expressed in a multitude of tissues (Catania et al., 2004) with varying levels of sequence homology between different receptors. For example, there is 38 % similarity between MC\(_2\) and MC\(_4\) and 60 % between MC\(_4\) and MC\(_5\). In addition to elevations in cAMP, \( \alpha \)-MSH can down-regulate NF-\( \kappa \)B activation by preventing phosphorylation and degradation of I\( \kappa \)B and thus cytokine synthesis (Figure 1.8) (Kelly et al., 2006). Increases in intracellular calcium and secondary activation of inositol tri-phosphate have also been proposed as potential mechanisms of action (Wikberg et al., 2000). In addition, PKC and/or PKA recognition sites have been identified on the melanocortin receptors, suggesting that MCs may act as activators of these signaling pathways (Chhajlani and Wikberg, 1992). A number of natural and synthetic melanocortin peptides have been isolated and shown to bind to the five MCs with different efficacies.
1.3.2.1 Melanocortin 1 Receptor (MC1).

MC1, a 317-amino-acid protein cloned in 1992 (Chhajlani and Wikberg, 1992, Mountjoy et al., 1994), is an intronless gene, located on chromosome 16q24.3. It has a multitude of functions including pigmentation, antipyresis and anti-inflammatory, due to its expression peripherally on melanocytes (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992), monocytes (Bhardwaj et al., 1997), neutrophils (Catania et al., 1996), endothelial cells (Hartmeyer et al., 1997), fibroblasts (Hill et al., 2006), mast cells (Adachi et al., 1999), lymphocytes (Neumann Andersen et al., 2001) and macrophages (Star et al., 1995) and within the central nervous system (Xia et al., 1995). A recent publication has unveiled that human chondrocytes express MC1 (Grassel et al., 2009). The receptor is activated by a number of endogenous peptides, with α-MSH being the most active followed by ACTH1-39, with β-MSH and γ-MSH causing weak activation (Getting, 2006). Truncated peptides ACTH4-10 and ACTH1-10 do not activate MC1, suggesting that both the amino and carboxyl-terminal ends of α-MSH (ACTH1-13) are important for full biological activation (Tatro, 1996). The relative affinity of melanocortin peptides for MC1 is shown in Table 1.2 below. Grässel and colleagues detected not only MC1 expression in articular chondrocytes in vitro, and in articular cartilage in situ, but they also found transcripts for MC2, MC5 and PCs, and confirmed these receptors were successfully translated into functionally active proteins, which upon activation modulate various pro-inflammatory cytokines, collagens and MMPs (Grässel et al., 2009).

1.3.2.2 Melanocortin 2 Receptor/ACTH receptor (MC2).

MC2 is an intronless gene, located on chromosome 18p11.2 and is translated into a 297-amino-acid long receptor (homo sapiens). It is unique among the other melanocortin receptors, as it is activated only by ACTH1-39, with no biological efficacy attributed to other melanocortin peptides (Getting, 2006). It has been detected in the adrenal cortex with expression within the zona fasciculata and the zone glomerulosa, the sites responsible for the synthesis and release of glucocorticoids and mineralcorticoids respectively (Mountjoy et al., 1992, Gantz and Fong, 2003) therefore it is essential for steroidogenesis. This receptor was also reported to be expressed in skin (Slominski et al., 1996) and in murine
adipocytes (*mus musculus*) (Boston and Cone, 1996) with a role in lipolysis when activated by ACTH (Boston, 1999). Mutations in this receptor are associated with the rare autosomal recessive disorder Familial Glucocorticoid Deficiency type 1, also known as hereditary unresponsiveness to ACTH (Clark et al., 1993).

1.3.2.3 Melanocortin 3 receptor (MC₃).

MC₃ is an intronless gene located on chromosome 20q13.2-q13.3 and its translation results in a protein, composed of 361 amino acids (*homo sapiens*) and 323 (*mus musculus*). MC₃ is expressed in the periphery and CNS (Gantz et al., 1993a), with ACTH₁-39, α, β and γ-MSH all equally potent in activating the receptor (Table 1.2).

In contrast to MC₁ the truncated peptides ACTH₄-10 and ACTH₁-10 are fully active at MC₃ (Gantz et al., 1993c), suggesting that the core region HFRRW is enough for activation. Expression was originally detected in the brain, placenta and the gut (Gantz et al., 1993a) but unlike the MC₁ and MC₂, no expression was detected in melanocytes or adrenal cortex (Gantz et al., 1993a). γ-MSH fails to induce an increase in circulating corticosterone, thereby it has been postulated that activation at MC₃ does not stimulate the hypothalamic-pituitary-adrenal (HPA) axis (Getting et al., 1999). Significant MC₃ expression has been reported within the hypothalamus and the limbic system, as well as the septum, hippocampus, thalamus and the midbrain (Roselli-Rehfuss et al., 1993). This pattern of expression has prompted the search of a central function played by MC₃ which appeared to be energy metabolism (Butler and Cone, 2003). Receptor expression has also been detected in the heart (Guarini et al., 2002, Getting et al., 2004), where it has been demonstrated to mediate myocardial protection (Getting et al., 2004) and in peritoneal (Getting et al., 1999, 2001) and knee joint MØ (Getting et al., 2002) whereby modulating immune response to inflammation (Getting, 2002). The latter is suggested following the observation that activation of MC₃ leads to an initial reduction in pro-inflammatory cytokines and chemokines, followed by the induction of anti-inflammatory mediators at later time points (Lam et al., 2005).
1.3.2.4 Melanocortin 4 receptor (MC4).

Human MC4 is located on chromosome 18q22 and is composed of 332 amino acids with certain similarities to peptides that activate the MC1 (Table 1.2). Expression appears to be restricted to the CNS since detailed studies of various peripheral organs have failed to detect this receptor subtype (Gantz et al., 1993b, Chhajlani, 1996). MC4 is more widely distributed than MC3 within the CNS, being found in the cortex, thalamus, hypothalamus, brain stem and the spinal cord (Mountjoy et al., 1994). The fact that PRO12 of ACTH1-39, shared by α-MSH, is not present in ACTH4-10 or γ-MSH suggests that this amino acid residue could be critical for the binding of MC4 (Gantz et al., 1993b). Physiologically, MC4 activation has been implicated in sexual dysfunction (Gantz and Fong, 2003), energy homeostasis, control of appetite (Butler and Cone, 2003) where the latter are of high interest within the pharmaceutical industry, due to the high demand for treatments of obesity.

1.3.2.5 Melanocortin 5 receptor (MC5).

MC5 was the last MC to be cloned (Gantz et al., 1994) and is located on chromosome 18p11.2, containing 325 amino acids (homo sapiens and mus musculus), with most potent agonists being α-MSH and ACTH1-39. Truncated peptides ACTH1-10 and ACTH4-13 have been found to provoke full activation of the receptor, although the effectiveness was considerably poorer when compared with α-MSH (Gantz et al., 1994). MC5 is widely expressed in peripheral tissues – liver, lung, thymus, testis, ovary, mammary glands, fat cells, bone marrow, skin, skeletal muscle, stomach and the duodenum (Gantz et al., 1994, Chhajlani, 1996). mRNA expression has also been detected in the olfactory bulb, substantia nigra and striatum of the rat CNS (Griffon et al., 1994). In addition, the expression of MC5 has been reported in B-lymphocytes (Buggy, 1998) and T lymphocytes (Taylor and Namba, 2001), a fact that demonstrates a potential role in immunomodulation. Other hypotheses on its physiological participation includes: sebaceous secretion, water repulsion, thermal regulation and exocrine gland control (Chen et al., 1997). However, the exact role of MC5 within the body needs to be further investigated.
α-MSH corresponds to the first 13 N-terminal amino acid residues of ACTH$_{1-39}$ and is identical in all mammals from which it has been isolated so far (e.g. *Mus musculus*, *Sus scrofa*, *Macaca mulata*, *Rattus norvegicus*, *Ovis aries*, *Camelus dromedarius*, *Equus caballus*). The N-terminal serine residue of α-MSH is often N-acetylated and the valine at the C-terminus is almost always covalently linked to a carboxamide group, modifications, rendering α-MSH more potent and stable against exopeptidase activities (Lerner and McGuire, 1964). The structure of β-MSH peptides of different vertebrates is more variable as compared to α-MSH. All β-MSH share 6 constant residues – Tyr$^5$, His$^9$-Phe$^{10}$-Arg$^{11}$-Trp$^{12}$ and Pro$^{15}$ and have an acidic iso-electric point (pl), as compared to α-MSH, which has higher pl. Conversely, γ-MSH sequence is not found in POMC precursor molecule of all vertebrate species. It is a dodecapeptide named Lys-$\gamma_1$-MSH or just γ-MSH, and in its longer form (22-31 aa) is termed γ$_3$-MSH. Significant sequence variations of the C-terminal sequence has been identified between species, whilst the N-terminal domain highly conserved (with Tyr$^2$, His$^6$-Phe$^7$-Agr$^8$-TRP$^9$ and Phe$^{12}$ being the invariant residues in all species). γ-MSH is processed from γ$_3$-MSH by cleavage at the dibasic residue pair RR (Figure 1.6), and contains an N-terminal lysine and carboxamide group that has been most likely generated similarly to those of α-MSH.

In addition to these endogenous melanocortin receptor agonists, two naturally occurring antagonists have been confirmed: the Agouti signalling protein (ASP) and agouti-related protein (AGRP). Agouti’s gene, which encodes a 131 aa protein containing a signal sequence, controls the relative amounts of eumelanin and phaeomelanin pigments in mammals. Lu and colleagues noticed that Agouti, produced in hair follicles, acts on follicular melanocytes to inhibit α-MSH elicited eumelanin production (Lu *et al*., 1994). What is more, it has been proven that agouti is a high-affinity antagonist to both MC$_1$ and MC$_4$ and acts by preventing α-MSH from activating adenylate cyclase (Lu *et al*., 1994), thus a role in inflammation has been proposed (Catania *et al*., 2004). It is now wildly known that ASP (the human equivalent of mouse agouti) is broadly distributed, with expressions in the foreskin, liver, kidney, adipose tissue, heart, testis, and ovary
(Wilson et al., 1995, Voisey and van Daal, 2002). Contrary, AGRP, is specifically expressed in the CNS – the neuronal cell bodies of the posterior hypothalamus in particular (Catania et al., 2004) and has been demonstrated to be an antagonist of MC3 (Ollmann et al., 1997). Additionally, AGRP blocked the anti-inflammatory effects of the selective MC3 agonist D[TRP]8-γ-MSH in a murine model of urate crystal peritonitis (Getting et al., 2006).

1.3.4 Synthetic melanocortins.

The melanocortin peptides are implicated in the regulation of pigmentation, adrenal function, memory, feeding behaviour, inflammation and more, as described previously (see section 1.3.2). Molecular cloning and sequencing of the melanocortin receptors has boosted systematic studies for investigation of the molecular mechanisms implicated in these functions. Synthetic and non-peptide development have enhanced our understanding of the biological functions of MCs in different tissues. The complexity and the wide expression distributions of the receptors, together with the high sequence homology has impeded the identification of all of their biological effects and has hindered the development of selective agonists and antagonists (Grieco et al., 2002). Identification of endogenous and synthetic peptides have been utilised to evaluate the pathophysiological role of the various MCs in different tissues. Extensive investigations led to the identification of the minimum fragment needed for activation of the MCs – HFRW, except MC2 (Wikberg et al., 2000). A number of peptides and non-peptide compounds have been developed over the last 20 years with varying degrees of selectivity to help elucidate the roles played by each receptor in disease pathologies.
<table>
<thead>
<tr>
<th>Melanocortin Receptor</th>
<th>Main site of expression</th>
<th>Ligand preference</th>
<th>Main physiological function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MC₁</strong></td>
<td>Melanocytes, macrophage, neutrophils, endothelial cells, fibroblasts, chondrocytes, CNS;</td>
<td>α-MSH= β-MSH= ACTH&gt;&gt; γ-MSH</td>
<td>Pigmentation, anti-pyretic and anti-inflammatory function;</td>
</tr>
<tr>
<td><strong>MC₂</strong></td>
<td>Adrenal cortex (zona fasciculata and zone glomerulata), skin;</td>
<td>ACTH only</td>
<td>Adrenal steroidogenesis;</td>
</tr>
<tr>
<td><strong>MC₃</strong></td>
<td>CNS, Placenta, stomach, pancreas, heart peritoneal, knee joint macrophages, chondrocytes;</td>
<td>γ-MSH = α-MSH = β-MSH = ACTH</td>
<td>Energy homeostasis, anti-inflammatory function, myocardial protection;</td>
</tr>
<tr>
<td><strong>MC₄</strong></td>
<td>CNS, spinal cord</td>
<td>α-MSH= β-MSH = ACTH &gt; γ-MSH</td>
<td>Appetite regulation, energy homeostasis, erectile function, pain;</td>
</tr>
<tr>
<td><strong>MC₅</strong></td>
<td>Lymphocytes, exocrine glands, skin, adrenal gland, adipose tissue, kidney, lymph nodes, liver, skeletal muscle.</td>
<td>α-MSH &gt; β-MSH = ACTH &gt; γ-MSH</td>
<td>Regulation of exocrine gland secretion.</td>
</tr>
</tbody>
</table>

**MC₁-₅** – melanocortin receptors; **ACTH** – adrenocorticotropic hormone; **MSH** – melanocortin stimulating hormone; **CNS** – central nervous system
1.3.4.1 Melanocortin receptor agonists.

[Nle⁴D-Phe⁷]-α-MSH (NDP-α-MSH) was one of the first synthesised analogues of α-MSH to display efficacy in inflammatory models (Sawyer et al., 1980), however, due to its length, the economic reality of bringing this peptide to market led to the development of smaller compounds. Given the short half-life and size of the peptide, smaller fragments were developed, including the potent MC₃/₄ lactam-based heptapeptide super agonist melanotan II (MTII: Ac-Nle-γ[Asp-DPhe-Lys]-NH₂; EC₅₀ = 2.8 nM) (Hadley et al., 1998) shown to be effective in models of murine obesity (Fan et al., 1997) and gouty arthritis (Getting et al., 1999, 2001).

Further identification of the natural agonists of the MC₃ led to the discovery that Tryptophan substitution at position 8 into the natural sequence of γ-MSH leads to a selective compound termed D[TRP]⁸γ-MSH (D[TRP]⁸γ-MSH: H-Tyr-Val-Met-Gly-His-Phe-Arg-DTrp-Asp-Arg-Phe-Gly-OH; EC₅₀ = 0.33 nM), which has been recognized to have a 300-fold and 250-fold improvement in selectivity for the MC₃ over MC₄ and MC₅, compared to the naturally occurring γ-MSH (EC₅₀ = 5.9 nM; Grieco et al., 2000).

These findings highlighted that those amino acids changes within the polypeptide of α-MSH lead to the development of increasingly selective compounds. The discovery of the synthetic selective MC₁ agonists MS05 (Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₃) and MS09 (Ser-Ser-Ile-Ile-Ser-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH₂) occurred following screening of peptide phage display library (Martignoni et al., 1997) with MS09 being more potent, but less selective than MS05 (Wikberg et al., 2000). These compounds potently down-regulate the expression and secretion of selectin (E-selectin), vascular cell adhesion molecule (VCAM), and intracellular endothelial cell adhesion molecules (ICAM) in human dermal vascular endothelial cells treated with TNF-α. In addition, they down-regulate IL-1β induced NF-κB activation in these cells (Brzoska et al., 1999). The octapeptide 154N-5 (Met-Phe-Arg-DTrp-Phe-Lys-Pro-Val-NH₂), which showed a high degree of HTB-14, HEK293, Jurkat, TNP-1 cell-lines, primary human cells and animal model (C3H/HEN mice) of TNF-α secretion (Ignar et al., 2003).
1.3.4.2  Melanocortin antagonists.

Development of SHU9119 is one of the major breakthroughs in melanocortin pharmacology. Substitution of the bulky aromatic amino acid DNa(2') into position 7 of ACTH1-39 led to the discovery of the antagonist at MC3 and MC4 with the structure Ac-Nle-c[Asp-His-DNa(2')-Arg-Trp-Lys]-NH2 (Hruby et al., 1995). SHU9119 has allowed many groups to dissect the role of MC3 in diseases ranging from arthritis to cardiovascular pathologies, and also in models of obesity as it has been shown to be an antagonist at MC3/4 in murine model of obesity (Fan et al., 1997; Butler et al., 2000) and models of acute inflammation (Hartmayer et al., 1997; Neumann-Andersen et al., 2001; Hill et al., 2006; Adachi et al., 1999; Getting et al., 1999, 2001). Other substitutions into positions 6 to 15 of ACTH1-39 have yielded the MC4 antagonist HS014 (Schioth et al., 1999), which blocked cAMP induced by α-MSH in MC4-transfected COS-1 cells. The selective cyclic MC4 antagonist HS024 (Kask et al., 1998) has an enhanced 10-fold potency compared to HS014 (Schioth et al., 1999) and has been demonstrated to stimulate food intake in rats (Kask et al., 1998). In order to investigate further the role of His6 in the core tetrapeptide of the melanocortin peptides, Grieco and colleagues designed, synthesized and characterized new cyclic lactam ligands with several modifications and the most important variant was the substitution of the Histidine residue at position 6 in SHU9119 with a Pro and Hyp (Grieco et al., 2002). This led to the discovery of the full agonists at MC5 – PG901 (Ac-Nle-c[Asp-Pro-DNa(2')-Arg-Trp]-Lys-NH2, EC50 = 0.072 nM), approx. 40 times more active than the superagonist MTII (EC50 = 2.8 nM), and PG911 (Ac-Nle-c[Asp-Hyp-DNa(2')-Arg-Trp]-Lys-NH2, EC50= 0.031 nM), which was synthesized by placing a Hyp residue at position 6). The His-Hyp substitution at position 6 of SHU9119 caused a remarkable agonistic activity of PG911 at MC5. In addition, both peptides showed noteworthy antagonistic activities on MC3 and MC4, compared to SHU9119 (Grieco et al., 2002).
1.3.5 Melanocortin peptides molecular mechanism of action.

The main intracellular signalling mechanism of stimulated melanocortin receptors is cAMP production via MC/Gs coupled to adenylate cyclase (Wikberg et al., 2000). However, the peptides have been shown to not only elicit cAMP increase, but also stimulate p38 pathway, extracellular signalling kinases (ERK; p44/42) (Mandrika et al., 2001), PKC and NF-κB transcription factors AP-1 and NF-κB as outlined in Figure 1.8 (Konda et al., 1994, Kalden et al., 1999, Ichiyama et al., 1999). The binding of melanocortins to its corresponding receptor is thought to activate protein kinase A (PKA), which has four main effects. First, PKA activation induces the phosphorylation of the cAMP-responsive-element-binding protein (CREB), which, owing to its high affinity for the co-activator CREB-binding protein (CBP), prevents the association of CBP with p65 (which is a key component of (NF-κB). Second, activated PKA inhibits IκB kinase (IKK), which stabilizes the IκB inhibitor and prevents nuclear translocation of p65. Third, PKA activation inhibits MAPK/ERK kinase kinase 1 (MEKK1) phosphorylation and activation, and the subsequent activation of p38 and TATA-binding protein (TBP). TBP need to be phosphorylated in order to be able to bind to the TATA box and to form an active trans-activating complex with CBP and NF-κB (Gonzalez-Rey et al., 2007). A reduction in the amounts of nuclear p65, CBP and phosphorylated TBP inhibits the formation of the conformationally active trans-activating complex that is required for the transcription of most cytokine and chemokine genes. Fourth, inhibition of MEKK1 by PKA subsequently deactivates Jun kinase (JNK) and c-Jun phosphorylation. The composition of the activator protein 1 (AP1) complex changes from the transcriptionally active c-Jun–c-Jun, to the transcriptionally inactive c-Jun–c-Fos or CREB (Gonzalez-Ray et al., 2007).

The final consequence is that the transcriptional machinery, which is perfectly assembled to the gene promoters of several inflammatory mediators (examples shown in red box) after the signalling of TNF-α through TNFR1, is significantly disrupted by treatment with melanocortin peptides. In addition to these effects of melanocortin peptides there is another mechanism of action that has been proposed. In human MC3 transfected HE LA cells, activation of cAMP-dependent PKA in the presence of α-MSH was shown to exert an inhibitory effect on the IP3-mediated increase in intracellular Ca2+ concentration (Konda et al., 1994).
The binding of melanocortins to its corresponding receptor increases cAMP and is thought to activate protein kinase A (PKA), which has four main effects. a) First, PKA activation induces the phosphorylation of the cAMP-responsive-element-binding protein (CREB) prevents the association of CBP with p65. b) The activated PKA hinders IκB kinase (IKK), which stabilizes the IκB inhibitor and stops nuclear translocation of p65. c) PKA activation prevents MAPK/ERK kinase kinase 1 (MEKK1) phosphorylation and activation, and activation of p38 and phosphorylation of the cAMP activating complex with CBP and NF-κB. d) Fourth, inhibition of MEKK1 by PKA subsequently deactivates JUN kinase (JNK) and cJUN phosphorylation. The composition of the activator protein 1 (AP1) complex changes from the transcriptionally active cJun–cJun, to the transcriptionally inactive JunB–cFos or CREB. e) The final consequence is that the transcriptional machinery is significantly disrupted by treatment with melanocortin peptides (Gonzalez-Ray et al., 2007).
1.4 Role of melanocortin peptides in arthritic pathologies.

1.4.1 Osteoarthritis.

Current treatment regimes for OA largely focus on exercise, surgery for joint replacement, and the treatment of symptoms, e.g., relieving pain with analgesics, rather than treating the underlying causes of the disease (Ettinger and Afable, 1994, Chen et al., 2008, Franklin et al., 2010).

Only a handful of studies so far have evaluated the therapeutic potential of ACTH and melanocortin peptides in OA. This may seem somewhat surprising at first glance given the number of studies suggesting therapeutic potential of these peptides in both pre-clinical models and human patient samples from RA and gouty arthritis patients. However, this could stem from the fact that whilst inflammation is considered causal to both RA and gouty arthritis, it is not generally accepted to be a major contributor to the development of OA.

Catania and colleagues evaluated the presence of systemic and synovial α-MSH in patients with OA and RA to evaluate whether changes in levels correlated with disease progression. α-MSH was detected in the synovial fluid of both OA and RA patients, with levels that were much lower in OA than RA (Catania et al., 1994). Of interest, however, was the observation that synovial levels of α-MSH were higher than plasma, suggesting a local production of this peptide (Catania et al., 1994). This could indicate that activation of resident cells within the joints cause the release of α-MSH, which in turn switches off disease progression in an auto or paracrine fashion. Again, given that inflammation is only considered to be a component of OA rather than a causal factor, this could explain why much lower levels of α-MSH are found in OA rather than RA patients. One potential mechanism for the articular cartilage degradation observed in OA may be due to TNF-α induced expression of MMPs, which can be down-regulated by α-MSH. In the human chondrosarcoma cell line HTB-94 (SW1353), α-MSH has been shown to down-regulate TNF-α induced expression of MMP13 mRNA and protein (Yoon et al., 2008). Studies with the pharmacological inhibitor SB203580, a p38 MAPK inhibitor, showed that α-MSH inhibited MMP13 by modulating p38 MAPK phosphorylation and subsequent activation of NF-κB (Yoon et al., 2008).
Although α-MSH has been shown to decrease TNF-α induced MMP expression, the parent hormone ACTH can cause terminal differentiation of chondrocytes and subsequent cartilage degeneration (Evans et al., 2004). Rodent chondrocytes and chondrocyte cell-lines express MC₃, matrix deposition was concentration dependently elevated in the presence of ACTH. These data highlight that the melanocortin system promotes chondrocyte phenotype development and their differentiation into mature chondrocytes, leading to an elevation in intracellular free calcium (Evans et al., 2005).

1.4.2 Rheumatoid arthritis.

RA is a complex pathology affecting many systems outside of the joints, with 40 % of RA deaths due to cardiovascular disease. Therapeutic intervention in RA is a leading cause of complications, for example NSAIDs may cause elevated blood pressure (Getting et al., 2009) and glucocorticoids increase cardiovascular problems by accelerating the rate of pathologies such as arterial thickening and narrowing (Getting et al., 2009). Methotrextate on the other hand may also promote heart disease by increasing levels of homocysteine. As a result of these complications and the fact that NSAIDs are contraindicated in the elderly due to high possibility of kidney failure, the progress of novel endogenous therapeutics is indispensable.

The role of melanocortin peptides in rheumatoid arthritis has not been fully ascertained yet. Catania et al. detected increased concentration of α-MSH in the synovial fluid of patient suffering from RA, juvenile arthritis, but not OA, that were greater than that detected in the plasma (Catania et al., 1994; Grässel et al., 2009) suggesting local production of the peptide, i.e. that the anti-cytokine molecule α-MSH is produced within a site of inflammation. This increase in α-MSH within synovial fluid also suggests the possibility of an endogenous anti-inflammatory loop maintaining a homeostatic balance within the joint and controlling the host inflammatory response. Joint concentrations of α-MSH in these patients were directly proportional to the degree of inflammation, whilst systemic (plasma) levels remained at physiological concentrations (Catania et al., 1994).
Utilising the adjuvant-induced arthritis model, α-MSH was shown to modulate weight loss, arthritic score, joint damage, and swelling, characteristic features of the disease. This effect on preventing weight loss was in contrast to that observed by the glucocorticoid prednisolone, which caused significant weight loss in these animals (Ceriani et al., 1994). The inflamed joint is characterised not only by infiltrating leukocytes, but also by activated resident bone/cartilage cells, such as osteoclasts and chondrocytes. Some evidence exists to suggest melanocortin receptor expression on these cells (Yoon et al., 2008). In situ hybridization shows that all MCs are expressed on chondrocytes in the mouse femoral bone and mRNA signals for all MCs, except MC1, were detected on primary rat osteoclasts, which also expressed the POMC gene (Zhong et al., 2005), thereby suggesting the possibility that POMC peptides generated by these cells could act in an autocrine/paracrine manner through their corresponding MCs. α-MSH has been shown to inhibit TNF-α-induced MMP13 by modulating p38 and NF-κB signaling in human chondrosarcoma cell-line HTB-94 (Yoon et al., 2008). These findings indicate that α-MSH may be beneficial in arthritic conditions.

1.4.3 Gouty arthritis.

ACTH1-39 was first shown to have clinical efficacy in gout in the 1950s, although no mechanism of action was described for its effects (Gutman and Yu, 1950). Long-term administration of ACTH1-39 causes a number of side effects, including suppression of the HPA axis, and thus its use was superseded by other therapies. Current treatments for gout focus largely on the use of colchicines, allopurinol and NSAIDs, which cause a number of side effects, including peptic ulceration, gastric problems, and are poorly tolerated in the elderly and patients with renal insufficiency (Ritter et al., 1994), a potential reason why there is a renewed interest in the use of ACTH1-39. Another reason is the fact that ACTH demonstrates a greater efficacy than conventional glucocorticoids in controlling gout (Ritter et al., 1994). The authors suggested the existence of another mechanism of action other than the well-characterised stimulation of ACTH/MC2 expressed on the adrenal gland (Ritter et al., 1994). Therefore, anti-inflammatory fragments of ACTH1-39 that do not activate the HPA axis have been sought in the
hope that they may have a better safety profile. Utilising murine models of gout, it has been demonstrated that smaller fragments of the POMC gene, including the peptides \( \alpha \)-MSH, \( \beta \)-MSH, and ACTH\(_{4-10} \), could reduce urate crystal–induced neutrophil migration, and pro-inflammatory cytokine and chemokine release (Getting et al., 1999). Of specific interest are the anti-inflammatory effects of these peptides, which occur in a corticosterone-independent manner and thus there is no reflex stimulation of the HPA axis.

These findings mirror those observed for ACTH\(_{4-10} \) in rat skin inflammation, where it was shown to inhibit prostaglandin generation and oedema formation (Gecse et al., 1980). The biological efficacy of these peptides was shown to occur via MC\(_3 \) expression on peritoneal macrophages with subsequent elevations in cAMP. Further confirmation of the role of MC\(_3 \) in mediating the anti-inflammatory effects of these peptides was highlighted when pre-treatment of mice with the MC\(_{3/4} \) antagonist SHU9119 led to an attenuation of their efficacy. Based on these findings, a hypothesis was proposed that MC\(_3 \) could be a novel therapeutic target for modulating the anti-inflammatory effects of the melanocortins, at least in this model of monosodium urate crystal deposition (Getting et al., 1999). Further studies promote this hypothesis with natural and synthetic agonists of the MC\(_3 \), \( \gamma_2 \)-MSH (Gecse et al., 1980) and MTII (Fan et al., 1997) respectively, attenuating inflammation both in vivo and in vitro (Getting et al., 2001). In a rodent model developed to mimic certain aspects of the human pathology, urate crystals were injected into the knee joint, which led to migration of neutrophils preceded by the release of IL-1\( \beta \) and IL-6. Local and systemic administration of the parent hormone ACTH\(_{1-39} \) resulted in significant inhibition of all parameters associated with joint inflammation (swelling, arthritic score, neutrophil migration) (Getting et al., 2002).

1.4.4 Role of melanocortins in other inflammatory pathologies.

In addition to the anti-inflammatory properties discussed above, melanocortins have been shown in various in vitro and in vivo experiments to possess modulatory roles in inflammation. In vitro, \( \alpha \)-MSH can reduce the T-lymphocyte co-stimulatory molecule CD86 in LPS stimulated monocytes (Bhardwaj et al., 1997), decrease LPS-induced TNF-\( \alpha \) production in whole blood (Catania et al.,
2000) and THP-1 cells (Taherzadeh et al., 1999) and inhibit NO synergistically elicited by LPS and IFN-γ in a MØ cell-line (Star et al., 1995, Mandrika et al., 2001). Supporting data were also shown in vivo in models of endotoxemia (Delgado Hernandez et al., 1999), inflammatory bowel diseases (Rajora et al., 1997b) and experimental heart transplant (Gatti et al., 2002), where melanocortin peptide treatment caused the reduction of NO and TNF-α.

Interestingly, α-MSH has been shown to modulate the elevation, production and release of the anti-inflammatory cytokine IL-10 in human peripheral blood mononuclear cells, thus promoting further the inhibition of pro-inflammatory cytokines induced by MØ (Bhardwaj et al., 1996). The importance of α-MSH-induced IL-10 in mediating anti-inflammatory responses was further substantiated in vivo, where the suppressive effect of α-MSH on airway inflammation is no longer observed in IL-10 knockout mice (Raap et al., 2003).

A remarkable facet of melanocortin biology is their ability to regulate peripheral acute inflammation following central administration. It was shown in brain ventricle injection that pre-treatment with α-MSH and ACTH11-13 reduced ear and hind paw oedema induced by local injection of IL-1, IL-8, leukotriene B4 and platelet-activating factor (Ceriani et al., 1994, Macaluso et al., 1994). When spinal cord trans-section was performed, these anti-oedema effects caused by central α-MSH injection was abolished, indicating that intact descending inhibitory neural pathways are needed for this effect (Macaluso et al., 1994).
1.5 **Aims of the study and hypothesis.**

The aims of this thesis were to examine the expression and functionality of melanocortin receptor MC₁ and MC₃ in human C-20/A4 cell-line and primary bovine articular chondrocytes. Another aim was to ascertain their anti-inflammatory, anti-apoptotic and chondroprotective effect on pro-inflammatory cytokine production following stimulation of *in vitro* cultures (C-20/A4 cell-line and primary articular chondrocytes) with TNFα/ mechanical impact of chondrocytes *in situ* and to investigate their effect on the production of anti-inflammatory proteins.

- To investigate the response of chondrocytes to inflammatory stimuli and the effect on pro-inflammatory cytokine (IL-1β IL-6, IL-8 and MCP-1), degradative matrix metalloproteinase (*MMP1*, *MMP3* and *MMP13*) and cartilage ECM matrix component (collagen type I and type II) production.

- To study the effect of classical anti-inflammatory drugs glucocorticoids (dexamethasone) and NSAIDs (indomethacin) on expression and release of IL-6, IL-8 and MMPs from activated chondrocytes.

- To ascertain melanocortin receptor expression, production and functionality in chondrocytes and determine whether melanocortin peptides can inhibit pro-inflammatory cytokines, and/or MMP production and induce anti-inflammatory proteins in cell-line and primary chondrocytes.

- To evaluate the protective properties of melanocortin peptides against TNF-α-induced apoptosis and caspases activity.

- To investigate the response of chondrocytes to lowered extracellular osmolarity, *e.g.* pro-inflammatory cytokines and degradative enzymes and the effect of melanocortin peptides on these parameters.

- To characterise the cytoprotective and anti-inflammatory effect of melanocortin peptides on mechanically impacted articular chondrocytes *in situ.*
Chapter 2
Materials and Methods
2.1 Materials and suppliers.

2.1.1 Chemical and reagents.

Table 2.1. Chemicals and Biochemicals used for tissue culture.

<table>
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<tr>
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<tr>
<td>Ascorbic acid</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>A4403</td>
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<td>Foetal Bovine Serum (FBS)</td>
<td>Invitrogen, Paisley, UK</td>
<td>16170078</td>
</tr>
<tr>
<td>GIBCO® DMEM + Glutamax™-1</td>
<td>Invitrogen, Paisley, UK</td>
<td>31966</td>
</tr>
<tr>
<td>Pen/Strep Solution (+5000 U/ml Penicillin, +5000 µg/ml Streptomycin)</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>P0781</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>Fisher Scientific, Leicestershire, UK</td>
<td>S13160/65</td>
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<tr>
<td>Trypsin-EDTA</td>
<td>Invitrogen, Paisley, UK</td>
<td>25300</td>
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Table 2.2. Chemicals and biochemicals used in protein extractions, SDS-PAGE and Western blots.

<table>
<thead>
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<th>Catalogue No.</th>
</tr>
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<td>Acryl Amide</td>
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<td>161-0100</td>
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<tr>
<td>Ammonium Persulfate</td>
<td>Bio-Rad Laboratories Ltd., Hertfordshire, UK</td>
<td>161-0700</td>
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<tr>
<td>β-mercaptoethanol</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>P0781</td>
</tr>
<tr>
<td>Bio-Rad Protein Assay (Bradford solution)</td>
<td>Bio-Rad Laboratories Ltd., Hertfordshire, UK</td>
<td>04693159001</td>
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<tr>
<td>Bis-acrylamide</td>
<td>Bio-Rad Laboratories Ltd., Hertfordshire, UK</td>
<td>161-0154</td>
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<tr>
<td>Coomassie® brilliant blue R250</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>25300</td>
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<tr>
<td>Dithiotreitol (DTT)</td>
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<td>BP172-25</td>
</tr>
<tr>
<td>Glycine, aminoacetic acid</td>
<td>Fisher Scientific, Leicestershire, UK</td>
<td>BPE381</td>
</tr>
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<td>Glycerol</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>49779</td>
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<tr>
<td>Methanol</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>32213</td>
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<tr>
<td>N,N,N′,N′-Tetramethylethylenediamine (TEMED)</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>T9281</td>
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<tr>
<td>NuPAGE® MOPS SDS Running Buffer 20x</td>
<td>Invitrogen, Paisley, UK</td>
<td>NP0001</td>
</tr>
<tr>
<td>NuPAGE® Transfer Buffer 20x</td>
<td>Invitrogen, Paisley, UK</td>
<td>NP0006</td>
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<tr>
<td>NuPAGE® Novex 10% Bis-Tris Gels</td>
<td>Invitrogen, Paisley, UK</td>
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Table 2.3 Reagents used in ELISAs.

<table>
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<td>3, 3’,5,5’–Tetramethylbenzidine tablets</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>T5525</td>
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<tr>
<td>Bovine albumin serum (BSA; fraction V, minimum 98%)</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>A2153</td>
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<tr>
<td>Dimethyl Sulfoxide (DMSO)</td>
<td>Fisher Scientific, Leicestershire, UK</td>
<td>D4121</td>
</tr>
<tr>
<td>Hydrogen Peroxide (30%)</td>
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<td>H1550</td>
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<tr>
<td>Phosphate Buffer Saline (PBS) tablets</td>
<td>Oxoid LPD, Hertfordshire</td>
<td>BR0014G</td>
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<tr>
<td>Phosphate Citrate Buffer</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>P4809</td>
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<td>Sodium acetate</td>
<td>Fisher Scientific, Leicestershire, UK</td>
<td>S210-500</td>
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<tr>
<td>Tween-20</td>
<td>Fisher Scientific, Leicestershire, UK</td>
<td>BPE337</td>
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Table 2.4 Primers used in this study.

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<th>Supplier</th>
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<td>β-actin forward and reverse</td>
<td>Eurofins MWG Operon, London, UK</td>
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<tr>
<td>Collagen I forward and reverse</td>
<td>Eurofins MWG Operon, London, UK</td>
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<tr>
<td>Collagen II forward and reverse</td>
<td>Eurofins MWG Operon, London, UK</td>
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<td>IL-6 forward and reverse</td>
<td>Eurofins MWG Operon, London, UK</td>
</tr>
<tr>
<td>IL-8 forward and reverse</td>
<td>Eurofins MWG Operon, London, UK</td>
</tr>
<tr>
<td>MMP1 forward and reverse</td>
<td>Eurofins MWG Operon, London, UK</td>
</tr>
<tr>
<td>MMP3 forward and reverse</td>
<td>Eurofins MWG Operon, London, UK</td>
</tr>
<tr>
<td>MMP13 Forward and reverse</td>
<td>Eurofins MWG Operon, London, UK</td>
</tr>
</tbody>
</table>

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Table 2.5. Antibodies used in this study.

<table>
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<th>Name</th>
<th>Supplier</th>
<th>Cat. No.</th>
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<tbody>
<tr>
<td>α-Tubulin Mouse mAb (clone B-5-1-2)</td>
<td>Sigma-Aldrich Company Ltd., Poole, UK</td>
<td>T5168</td>
</tr>
<tr>
<td>Cleaved Caspase-3 (Asp175) (5P1) Rabbit mAb</td>
<td>Cell Signalling Technology Inc., UK</td>
<td>9664</td>
</tr>
<tr>
<td>Goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG</td>
<td>Dako Cytomation Ltd., Cambridgeshie, UK</td>
<td>P0448</td>
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<tr>
<td>Goat anti-mouse horseradish peroxidase (HRP) conjugated IgG</td>
<td>Dako Cytomation Ltd., Cambridgeshie, UK</td>
<td>P0447</td>
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<tr>
<td>Heme oxygenase(HO)-1 rabbit pAb</td>
<td>Bioquote Ltd. (supplier for Stressgen Biotechnologies), York, UK.</td>
<td>SPC-112C</td>
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Table 2.6 Commercial Kits used in this study.

<table>
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<tr>
<th>Commercial Kits</th>
<th>Supplier</th>
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</tr>
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<tr>
<td>cAMP Biotrak™ EIA system</td>
<td>GE Healthcare UK Ltd., Buckinghamshire, UK</td>
<td>RPN2251</td>
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<tr>
<td>Caspase-Glo® 3/7</td>
<td>Promega UK Ltd., UK</td>
<td>TB323</td>
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<tr>
<td>DuoSet® ELISA Development systems</td>
<td>R&amp;D Systems® Europe Ltd., Abingdon, UK</td>
<td>N/A</td>
</tr>
<tr>
<td>GoTaq Green Mastermix 2x</td>
<td>Promega UK Ltd., Southampton, UK</td>
<td>M7122</td>
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<tr>
<td>ImProm–II™ Reverse Transcription System</td>
<td>Promega UK Ltd., Southampton, UK</td>
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<tr>
<td>NucleoSpin® RNA/Protein</td>
<td>GE Healthcare UK Ltd., Buckinghamshire, UK</td>
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<tr>
<td>Parameter™ PGE₂ Assay</td>
<td>R&amp;D Systems® Europe Ltd., Abingdon, UK</td>
<td>KGE004B</td>
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<td>Materials</td>
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<td>-----------------------------------</td>
<td>----------------------------------------------</td>
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<td>α-MSH; ACTH₁₋₁₃</td>
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<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)</td>
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<td>3-isobutyl-1-methylxanthine (IBMX)</td>
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<td>Calcein-AM</td>
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<td>Sigma–Aldrich Inc. Poole, UK</td>
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<tr>
<td>D[TRP]⁸-γ-MSH</td>
<td>Kind gift by Prof. Paolo Grieco, University of Naples</td>
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<td>Ethidium bromide (EtBr₂)</td>
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<td>Forskolin</td>
<td>Sigma–Aldrich Inc. Poole, UK</td>
<td>F6886</td>
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<td>FUJI Medical X–Ray Film</td>
<td>FUJIFILM EUROPE GmbH, Düsseldorf, Germany</td>
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<td>Full–range Rainbow™ Molecular Weight Marker</td>
<td>GE Healthcare UK Ltd., Buckinghamshire, UK</td>
<td>RPN800</td>
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<td>Hybond™–C Extra Nitrocellulose membrane</td>
<td>Amersham Biosciences UK Ltd. Buck, UK</td>
<td>RPN303E</td>
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<td>Hydrochloric acid (HCl)</td>
<td>VWR International (supplier for BDH), Leicestershire, UK</td>
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<td>Indomethacin</td>
<td>Sigma–Aldrich Inc. Poole, UK</td>
<td>I7378</td>
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<td>Interleukin(IL)-6</td>
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<td>SRP4145</td>
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2.1.2 Software.

<table>
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<td>Image J</td>
<td>Bitplane AG, Zurich, Switzerland</td>
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<td>Imaris 7.1.1</td>
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<tr>
<td>Microsoft Office 2008</td>
<td>Microsoft, USA</td>
</tr>
<tr>
<td>GraphPad Prism</td>
<td>GraphPad Software, San Francisco, USA</td>
</tr>
<tr>
<td>OmniGraffle Pro 5.02</td>
<td>Washington, USA</td>
</tr>
<tr>
<td>Leica TCS SP1/SP2</td>
<td>Leica Microsystems (UK) Ltd., Milton Keynes UK</td>
</tr>
<tr>
<td>EndNote X2</td>
<td>Adept Scientific, Herts, UK</td>
</tr>
</tbody>
</table>

Table 2.8. Software used in this study.
2.2 Methods.

2.2.1 Cell line storage and resuscitation.

C-20/A4 is a human chondrocytic cell line, derived from juvenile costal chondrocytes (Finger et al., 2004). C20/A4 chondrocytes were stored in liquid nitrogen in 1.0 ml aliquots of 1.0 x 10^6 cells/ml in Dulbecco Modified Eagle Medium (DMEM) supplemented with 50.0 % FCS and 20% DMSO. Prior to cell culture, cells were rapidly thawed at 37°C in a water bath and pre-warmed complemented medium (DMEM + Glutamax supplemented with 50 U/ml Penicillin, 50 µg/ml Streptomycin and 10% FCS) added to dilute the toxicity of the DMSO. The resulting cell suspension was centrifuged at 2000 rpm for 10 min and supernatant discarded, thus removing DMSO. The cell pellet was then resuspended in complete medium, transferred into 25 cm^2 flasks and incubated in humidified incubator with 95% air : 5% CO₂ to allow cell proliferation.

2.2.2 Cell line culture and maintenance.

Cell culture media was replaced at 4-day intervals whereby media were removed by aspiration and replaced by fresh complemented DMEM. At 80 % confluence, chondrocytes were subcultured by lifting the cells from their substrate using 0.05 % Trypsin-EDTA and centrifuging of detached adherent cells at 2000 rpm for 10 min. The resulting cell pellet was resuspended and seeded in new culture flasks (1.0 x 10^3 cells/cm^2) for continuous culture or in various (6-, 24-, or 96-) well plates (1.0 x 10^5 cell/cm^2) as appropriate for experimentation. Cells were incubated at 37 °C in a humidified atmosphere supplied with 95% air : 5% CO₂. Cell line passage numbers used in all experiments were between 1 and 10.

2.2.3 Cartilage removal and chondrocyte isolation.

Fresh metacarpal or metatarsal phalangeal joints of 18 – 21 month old female animals were obtained with permission from the local abattoir and were dissected under aseptic conditions on the day of slaughter. Full depth articular cartilage, excluding subchondral bone, was removed carefully from the load bearing surfaces of the metacarpal phalangeal joints and cartilage explants cultured in DMEM (serum-free; 280 mOsm/kg:H₂O; 37°C, 95% air :5% CO₂) until required
(used within 96h). Osmolarity was measured using Vapro™ vapour pressure osmometer and accurate to ± 5 mOsm (Kerrigan and Hall, 2008).

For chondrocyte isolation, cartilage explants were incubated for ~18h at 37ºC with 0.8 mg/ml Collagenase in DMEM 380 mOsm (Bush and Hall, 2001). The osmolarity of the DMEM was raised by addition of 3.14 mg/ml filter-sterilized NaCl (using 2.0 µm filter) to limit cell swelling upon chondrocytes released from the matrix (Hall et al., 1996b). Digest material was passed through a tea strainer, and 20.0 µm nylon filters (Falcon) to remove undigested material and any large cell clumps. Chondrocytes were washed 3x by centrifugation (8 min, 20º C, 600g) in DMEM (380 mOsm/kg:H2O). For stimulation of freshly isolated bovine chondrocytes (P0 – passage 0), chondrocytes were seeded at 1.0 x 10^5 cells/cm² in 25 cm² plastic flasks in the same serum-free 380 mOsm DMEM for 2 – 24 h to allow for cell attachment.

For long-term culture of primary bovine chondrocytes, cells were isolated as described above and following the third wash, chondrocytes were resuspended in ‘complemented’ DMEM (380 mOsm/kg:H2O; 10% FCS, 50 µg/ml ascorbic acid, 50 U/ml Penicillin, 50 µg/ml Streptomycin; pH 7.4; Kerrigan et al., 2008). Media was replaced when required and chondrocytes were subcultured up to passage 3 (P3; 21 days). Briefly, supplemented media was removed and chondrocytes were lifted from the plastic using Trypsin-EDTA 0.05%. The suspension was diluted 1:2.5 in DMEM (380 mOsm) prior to washing by centrifugation (3x). Subsequently, cells were seeded as described above in plastic flasks with various sizes (depending on the experimental needs).

2.2.4 Application of single impact to bovine articular cartilage explants.

A vertical drop tower was used to deliver single blunt impact to individual cartilage explants under aseptic conditions. The impacting weight and the base were made of hard nylon with smoothly polished surface as previously described (Bush et al., 2005). The individual cartilage explants were carefully positioned with the synovial surface uppermost, and exposed to single blunt impact with a weight of 137 g dropped from a height of 10 cm, equivalent to 1.14 N, 6.47 kPa (assuming linear acceleration). Multiple impacts occurred rarely, and these
Explants were discarded. The cartilage pieces were then removed from the drop tower and returned to the 10 cm² dishes for 6 h when the chondrocytes were incubated for 30 min with Calcein-AM and visualised under confocal laser scanning microscopy (CLSM). Human knee forces are approximately 12.0 kPa, significantly higher than the ones used here (Matthews et al., 1977), but it has to be noted that the articular cartilage explant did not have the support of the underlining bone, or the protection of the synovial fluid available in vivo. Therefore, the impact tower was used for injuring the cartilage and not as a model of forces found in vivo.

Explants of bovine articular cartilage were incubated for 30 min with or without melanocortin peptides α-MSH and D[TRP]β-γ-MSH (3.0 μg/ml) prior to single impact and then incubated for 6 h. Explants were then incubated with the fluorescent indicators Calcein-AM (5.0 μM) and Propidium Iodide (1.0 μM) permitting the measurement of cell viability using CLSM.

Cell viability of in situ chondrocytes was investigated by CLSM and cytokine release (IL-1β, IL-6, IL-8, MCP-1) from cartilage explants by ELISA. Tissue was viewed by placing the cartilage piece in a new dry dish and viewing perpendicularly to the articular (synovial) surface. A small amount of cyanoacrylate glue was applied to a small corner of the explant, at a distant point of that to be viewed and PBS was added.
2.2.5 Confocal Microscopy.

2.2.5.1 Confocal Microscopy Imaging.

Images were taken using an upright Leica TCS NT CLSM (Leica Microsystems, Milton Keynes, UK) and Leica Software. The following parameters were adopted for the acquisition of the images (Kerrigan and Hall, 2005).

- Grey resolution of 8 bit
- Pinhole: 1.00 airy unit, matched to the diameter of objective used
- Beam splitter 488/543/633
- Laser speed 400 Hz
- Dipping lens HCX APO L U-V-1 63.0x 90 W

During the incubation of cartilage explants with Calcein-AM and PI, the molecules diffused through the matrix into the cells.

Calcein-AM is non-fluorescent when outside the cell. It is cell membrane permeable and once in the cell, the molecule undergoes enzymatic cleavage by intracellular esterase’s, a process, which releases the fluorophore – Calcein. It is comparatively membrane impermeable and therefore is trapped within the cells. After excitation of the dye with a 488 nm argon laser (emission at 525 nm), Calcein fluoresced, providing useful indication of cytoplasmic space in the living cells. Propidium Iodide (PI) is membrane impermeable and only when bound to nucleic acids, it fluoresced red (excitation at 525 – 535 nm and emission = 650 – 670 nm), therefore indicating the presence of dead cells. When cartilage was impacted, it was possible to observe cell death, as dying cells lost their green fluorescence rapidly as it leaked out of cell membranes upon impact. Instead, they fluoresced red, as the PI entered the cell through the leaky membrane and bound to the nucleic acids.

Chondrocytes in cartilage explants were viewed by an upright Leica TCS SP2 CLSM (Leica Microsystems, Milton Keynes, UK) with low power magnification of x10 air objective lenses for cell viability measurements. Laser power and detector sensitivity were adjusted to provide optimal image quality, preventing dye
bleaching (Bush and Hall, 2001a,b). Scanning speed was 0.6 Hz with double frame integration, double line averaging for 1024x1024 pixel image.

2.2.5.2 Image analysis.

Confocal microscopy collects a series of planar images comprised of voxels (3D pixels). Each voxel contains information about its relative position and intensity. To be able to correctly portray a cell, it was essential to ascertain the correct boundaries of each individual cell. Images of chondrocytes loaded with Calcein-AM showed strong homogenous fluorescence that differed tremendously from the emission of the surrounding matrix/DMEM media, which shows negligible fluorescence under the conditions used for this thesis.

In order to determine cell viability prior to and post impact, CLSM data were imported into and analysed using Imaris 7.1.1 Spots feature. Based on the average diameter of viable cells (green) and “dead” cells (red), in the cartilage explant, spot analysis was used to calculate the total number of cells and the number of viable cells within the area of observation. For spots analysis, the optimum threshold for Calcein-AM and PI was calculated by selecting a smaller area with a known number of cells and different threshold values from 0 – 100% were applied in increments of 10 %. Using the resulted linear regression formula, the correct cell number was determined with a threshold percentage of 20 % for Calcein-AM-stained cells (viable cells) and 60 % for PI-stained cells (dead cells) as shown on Figure 2.2.
Figure 2.1 Schematic representation of 3D image reconstitution using Imaris 7.1.1. A series of images were taken using upright Leica CLSM along the z-axis of chondrocytes adherent to the surface of a plastic 10 cm$^2$ culture dish (A). The images were imported in Imaris 7.1.1 and reconstituted (B) to give a 3D view of the observed cells (volume objects; C). Using the Surfaces visualization feature of the software, the correct cell boundaries above the background cut-off were identified by a computer-generated representation of a specified grey value range in the data set. It creates an artificial solid object (D) in order to visualize the range of interest of the volume object.
Figure 2.2 Optimisation of spot analysis.
Cartilage explants were stained with Calcein-AM (5.0 µM) and Propidium Iodide (1.0 µM) and imaged under CLSM. An area of visually countable cells (Panel B) was cropped from the 3D CLSM image (Panel A) and spot analysis was applied to the cropped section (Panel C) for both the viable (green) and non-viable (red) cells by altering the baseline threshold in 10 % increments from 0-100 %. Actual cell number was used to accurately choose the optimal threshold percentage for Calcein-AM and PI.
2.2.6  Cell-line and primary bovine chondrocyte stimulation.

2.2.6.1  *In vitro* cell stimulation with various inflammogens.

Primary bovine chondrocytes P₀ and C-20/A4 chondrocytes seeded in 96-well plates were stimulated with TNF-α (20.0 – 80.0 pg/ml), LPS (0.1 – 10.0 µg/ml), MSU crystals (0.1 – 10.0 µg/ml), H₂O₂ (0.01% - 1.0%). Both control and stimulated chondrocytes were incubated for various time periods, outlined in figure legends, in humidified incubator at 37°C, 95% : 5% air : CO₂. Cells were analysed for MTT reduction to assay cell viability and media collected for Griess nitrite accumulation assay and cytokine ELISAs (for detailed protocols, see: sections 2.3.4; 2.3.1 and 2.3.2 respectively).

2.2.6.2  Chondrocyte drug treatment.

2.2.6.2.1  *In vitro* melanocortin agonist treatment of human C-20/A4 and primary P₀ bovine articular chondrocytes.

C-20/A4 cell line or primary bovine P₀ chondrocytes were seeded in 6- 24- or 96-well plates and incubated for 24h at 37°C in humidified incubator at 37°C, 95% : 5% air : CO₂ to allow cell adhesion to the culture substrate. Culture medium was then replaced with serum-free DMEM medium and chondrocytes incubated in different conditions for various time points (outlined in the respective figure legends). Chondrocytes were treated with a panel of melanocortin peptides – α-MSH, D[TRP]⁸-γ-MSH, PG901, PG911, or SHU9119 in a concentration range (0.1 – 30.0 µg/ml) for 2 – 24 h in serum-free DMEM ± TNF-α (60.0 pg/ml) for C-20/A4 cells or for 6 h only (P₀ bovine cells). The cells in the 24-well plates and 96-well plates were analysed for MTT and cell-culture media were collected for cytokine ELISA experiments.

In separate experiments, cells were treated with α-MSH, D[TRP]⁸-γ-MSH (3.0 µg/ml) or SHU9119 (10.0 µg/ml) ± TNF-α (60.0 pg/ml) for 6 h. For protein expression and RNA extraction, cells were removed from the 6-well plates with a cell scraper and the respective experiment was performed as described in individual figure legends. The cells in the 24-well plates and 96-well plates were
analysed by MTT assay for cell viability, and cell-culture media were collected for cytokine ELISA experiments.

Alternatively, cells were treated with $\alpha$-MSH, D[TRP]$^8$-$\gamma$-MSH (3.0 µg/ml) ± SHU9119 (10.0 µg/ml) + TNF-$\alpha$ (60.0 pg/ml) for 6 h and the media from 24-well plates were used for cytokine ELISA measurements. Cells plated in 6-well plates were used for RNA extraction.

2.2.6.2.2 In vitro dexamethasone and Indomethacin treatment of human C-20/A4 chondrocytes.

To investigate the effect of classical anti-inflammatory drugs in this system, two widely used drugs were chosen dexamethasone (glucocorticoid) and Indomethacin (NSAID). Human C-20/A4 chondrocytes were pre-treated for 30 min with 1.0 µM of either dexamethasone or indomethacin and then stimulated with TNF-$\alpha$ (60.0 pg/ml) for 0 – 24 h. Total RNA was extracted and reverse transcribed into cDNA. Conventional PCR was employed to investigate the transcription levels of IL6 and IL8 at time point 6 h. From the same experimental set-up, cell-free supernatants were collected at time points 0, 2, 4, 6 and 24 h and analysed for IL-6 and IL-8 protein release via commercially available ELISAs.

2.2.6.2.3 In situ cartilage explants treatment with melanocortin peptides and dexamethasone.

Cartilage explants were obtained as explained earlier (Section 2.2.3) and treated with PBS, dexamethasone (1.0 µM) or melanocortin peptides – $\alpha$-MSH/D[TRP]$^8$-$\gamma$-MSH (3.0 µg/ml) for 6 h prior to confocal microscopy for evaluation of their effect on chondrocyte viability. From the same experimental set-up, cell free supernatants were collected to evaluate basal release of IL-1$\beta$, IL-6 and IL-8 and to compare that to the effect of the abovementioned agents.

Alternatively, in order to evaluate and compare the protective properties of the melanocortin peptides and steroids on mechanically injured cartilage explants, the cartilage pieces were incubated for 30 min with PBS (control), dexamethasone (1.0 µM) or melanocortin peptides – $\alpha$-MSH/D[TRP]$^8$-$\gamma$-MSH (3.0 µg/ml) ± SHU9119 (10.0 µg/ml) + TNF-$\alpha$ (60.0 pg/ml) for 6 h and the media from 24-well plates were used for cytokine ELISA measurements.
µg/ml) for 30 mins prior to cartilage impact (using drop tower as explained in section 2.2.4). The cartilage explants were then returned to their vials immediately, whereby they were incubated for additional 6 h at 37°C prior to confocal microscopy visualization for identification of the effect of the treatments on cell death and changes in chondrocyte morphology. Cell-free supernatants were collected at that time-point in order to evaluate: 1.) the effect of mechanical trauma on pro-inflammatory cytokine production by the injured chondrocytes, and 2.) the therapeutic, anti-inflammatory effect of dexamethasone, α-MSH and D[TRP]8-γ-MSH on chondrocytes following mechanical trauma.

2.2.7 mRNA and protein analysis

2.2.7.1 RNA purification and quantification.

Total RNA was extracted from primary and cell-line chondrocytes using the commercially available NucleoSpin® RNA II Kit. Cell culture medium was aspirated and the cells washed with PBS, prior to trypsinizing them using 0.05 % Trypsin. Cell suspension was centrifuged at 3000 rpm for 5 min and subsequently lysed by incubation of the cell pellet with 350 µl buffer RA1 (containing 3.5 µl β-ME), which contains large amounts of chaotropic ions, therefore immediately inactivating RNases, creating appropriate binding conditions favoring adsorption of RNA to the silica membrane. The viscosity of the lysate was reduced by centrifugation at 13,000 rpm for 1 min. RNA binding conditions were adjusted by the addition of 350 µl of 70 % EtOH to the filtrated lysate and mixed by vortexing (2 x 5 s). The mixture was then loaded onto a NucleoSpin RNA II Column and RNA bound to the silica membrane by centrifuging the lysate at 13,000 rpm for 1 min. 350 µl Membrane Desalting Buffer were applied to the membrane and centrifuged at 13,000 rpm for 1 min, improving the efficacy prior to rDNase digestion by salt removal.

Contaminating DNA bound to the silica membrane, was removed by rDNase solution, directly applied to the membrane during the preparation and incubated at room temperature for 15 min. The sample was washed with buffer RA2 to inactivate the rDNase and subsequently washed twice with buffer RA3. Pure RNA was finally eluted under low ionic strength conditions with RNase-free H₂O.
The concentration of samples was then determined by measuring the absorbance at $A_{230}$, $A_{260}$ and $A_{280}$ using NanoDrop® ND-1000 UV-Vis Spectrophotometer, which enables highly accurate analyses of extremely small samples with remarkable reproducibility. Readings were deemed significant if $A_{260} \geq 0.1$ (1 absorbance unit at 260 nm corresponds to 40.0 µg/ml RNA). The $A_{260}/A_{280}$ and the $A_{260}/A_{230}$ ratios provided an estimate of the purity of the RNA, which was considered uncontaminated if both absorbance ratios were in the range of 1.9 – 2.1.

### 2.2.7.2 Complementary DNA (cDNA) synthesis.

cDNA was synthesized by mixing 1.0 µg of DNase-treated total RNA, PolyT and Random Primers in a total volume of 20.0 µl (Promega) using the Improm II Reverse Transcription System. Briefly, RNA and the oligonucleotides were incubated at 70°C for 5 min to allow denaturation of RNA secondary structures and primers were hybridized to the RNA by cooling the samples to 4°C for 5 min. Reaction buffer, MgCl$_2$, dNTPs and Reverse Transcriptase (RT) were then added and incubated at 42°C for $\geq$ 1 h and the RT was heat inactivated at 70°C for 15 min.
2.2.7.3 Polymerase Chain Reaction (PCR).

Polymerase chain reaction (PCR) is a method used to amplify a specific DNA sequence in vitro by repeated cycles of synthesis with specific primers and thermotolerant DNA polymerase (Saiki et al., 1988). Specific primers are complementary to sequences that lie on opposite strands of the template DNA and flank the segment of DNA to be amplified. Template DNA was first denatured by heating the reaction to 95°C in the presence of a large molar excess of each of the two primers and dNTPs. The reaction mixture was cooled to allow the primers to anneal to their target sequences, and subsequently the annealed primers extended at 72°C by Taq DNA polymerase.

Primer sequences for each gene, together with their specific T_m and the resulting fragment size are outlined in Table 2.9. Synthetic oligonucleotides were purchased from MWG Biotech AG, Ebersberg, Germany. Cycles of denaturation, annealing, and DNA synthesis were repeated 25 times as outlined below (Table 2.10). The PCR with Taq polymerase was used for gene screening procedures, where the expression of key genes was checked with specific primers. GoTaq®Green Master Mix (Promega) and cDNA amplification performed with annealing temperature set to be equal to that of the primer with lower T_m.
<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide Sequence</th>
<th>Length [bp]</th>
<th>Tm [°C]</th>
<th>Fragment Size [bp]</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin_FWD</td>
<td>GTC CCG GCA TGT GCA A</td>
<td>16</td>
<td>54.3</td>
<td>550</td>
</tr>
<tr>
<td>β-actin_REV</td>
<td>AGG ATG TTC ATG AGG TAG T</td>
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<td>52.4</td>
<td></td>
</tr>
<tr>
<td>MC1R_FWD</td>
<td>GCT GGA CAA TGT CAT TGA CG</td>
<td>20</td>
<td>57.3</td>
<td>497</td>
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<tr>
<td>MC1R_REV</td>
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<td>54.5</td>
<td></td>
</tr>
<tr>
<td>MC3R_FWD</td>
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<td>56.7</td>
<td>820</td>
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<td>MC3R_REV</td>
<td>CCA GCA GAA GAT GAA CAC</td>
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<td>53.7</td>
<td></td>
</tr>
<tr>
<td>MC5R_FWD</td>
<td>GCC ATC ACG CCG GCA TC</td>
<td>17</td>
<td>58.7</td>
<td>340</td>
</tr>
<tr>
<td>MC5R_REV</td>
<td>GAG ACA TGA AGC GAG AGC</td>
<td>18</td>
<td>71.6</td>
<td></td>
</tr>
<tr>
<td>GAPD_FWD_BOV</td>
<td>AGA ACG GGA AGC TTG TCA TC</td>
<td>20</td>
<td>57.0</td>
<td>743</td>
</tr>
<tr>
<td>GAPD_REV_BOV</td>
<td>TGA GCT TGA CAA AGT GGT CGT</td>
<td>21</td>
<td>58.0</td>
<td></td>
</tr>
<tr>
<td>MC1R_FWD_BOV</td>
<td>CCA CCC TCC CCT TCA CCC TGG</td>
<td>21</td>
<td>67.6</td>
<td>301</td>
</tr>
<tr>
<td>MC1R_REV_BOV</td>
<td>CAT TGT CCA GCT GCT GCA CCA CG</td>
<td>23</td>
<td>66.0</td>
<td></td>
</tr>
<tr>
<td>MC5R_FWD_BOV</td>
<td>GTC CAG AAT GCA TCC TCA CTA TGT GAG G</td>
<td>28</td>
<td>66.6</td>
<td>642</td>
</tr>
<tr>
<td>MC5R_REV_BOV</td>
<td>CAG GGT GAT GGC GCC CTT CAC G</td>
<td>22</td>
<td>68.0</td>
<td></td>
</tr>
<tr>
<td>IL6_FWD</td>
<td>CTC AGC CCT GAG AAA GGA GA</td>
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<td>IL6_REV</td>
<td>TGC AGG AAC TCC TTA AAG CTG</td>
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<tr>
<td>IL8_FWD</td>
<td>ATG ACT TCC AAG CTG GCC GTC</td>
<td>24</td>
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<td>299</td>
</tr>
<tr>
<td>IL8_REV</td>
<td>TTA TGA ATT CTC AGC CCT CTT CAA AAA</td>
<td>27</td>
<td>58.9</td>
<td></td>
</tr>
<tr>
<td>MMP1_FWD</td>
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<td>61.3</td>
<td>786</td>
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<tr>
<td>MMP1_REV</td>
<td>AAG GTT AGC TTA CTG TCA CAC ACG CTT</td>
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<td>63.4</td>
<td></td>
</tr>
<tr>
<td>MMP3_FWD</td>
<td>GGA AAT CAG TTC TGG GCT ATAGA CGA GG</td>
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<td>64.8</td>
<td>301</td>
</tr>
<tr>
<td>MMP3_REV</td>
<td>CCA ACT GCG AAG ATC CAC TGA AGA AG</td>
<td>26</td>
<td>64.8</td>
<td></td>
</tr>
<tr>
<td>Component</td>
<td>Volume (µl)</td>
<td>Final Conc.</td>
<td>PCR Step</td>
<td>Time (min)</td>
</tr>
<tr>
<td>--------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>GoTaq Green</td>
<td>12.5</td>
<td>1x</td>
<td>Initial denaturation</td>
<td>5:00</td>
</tr>
<tr>
<td>Mastermix, 2x</td>
<td></td>
<td></td>
<td>Denaturation</td>
<td>1:00</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>2.0</td>
<td>1 µM</td>
<td>Annealing</td>
<td>1:30</td>
</tr>
<tr>
<td>10 µM</td>
<td></td>
<td></td>
<td>Elongation</td>
<td>1:30</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2.0</td>
<td>1 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cDNA Template</td>
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<td>&lt;250 ng</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclease-Free</td>
<td>6.5</td>
<td>N/A</td>
<td>Final Elongation</td>
<td>10:00</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Volume</td>
<td>25</td>
<td>N/A</td>
<td>Soak</td>
<td>∞</td>
</tr>
</tbody>
</table>

Table 2.10. Master mix and program for PCR with Taq polymerase.
2.2.7.4 DNA separation by agarose electrophoresis.

Linear double-stranded DNA-fragments were separated by an electric field due to the fact that the migration of DNA varies as the reciprocal of the logarithm of the base-pairs number (Meyers et al., 1976). Upon addition of ethidium bromide, which is intercalated into GC-pairs, DNA becomes fluorescent and can be visualized under UV-light. Agarose gels (2.0 %) were prepared in TBE buffer (40 mM Tris-acetate, 1.3 mM EDTA–Na, 0.47 mM CH₃COOH). Agarose gels (2 %) were prepared in TBE–buffer and DNA samples loaded onto the gel together with positive (β–actin) and negative controls and separated at 80–150 V. DNA fragments’ size was determined by comparing to a DirectLoad™Step Ladder 50 bp (Sigma-Aldrich, Poole, Dorset, UK), suitable to estimate the size of DNA fragments in the range of 50–3000 bp. After electrophoresis the gel was stained in ethidium bromide (10.0 µg/ml) in TBE-buffer for 5-15 min. and visualized under UV light.

2.2.7.5 Protein extraction.

There are two broad categories for complementary analytical applications of extracted proteins: identification and quantification studies and functional studies, including enzymatic assays and binding studies. Here, quantification studies have been carried out in order to determine the amount of proteins following stimulation of chondrocytes with various agents. Quantity determination is based on protein sequencing or antibody binding and does not require preservation of secondary, tertiary and quaternary structures.

Following stimulation of C-20/A4 cells with various stimuli (detailed explanation in 2.2.6.2) cell supernatants were removed and 0.5 ml of hot lysis buffer (10% v/v glycerol, 62.5 mM Tris–HCl – pH 6.8, 2% SDS, 100 mM DTT; 65° C) was added to each well. Cells were removed by scraping them off the plate using a cell scraper and lysate was collected and passed through a 25 gauge needle in order to disrupt the cell membranes. This method denatures the proteins by solubilisation with the anionic detergent Sodium Dodecyl Sulphate (SDS). Protein extracts were separated on SDS–PAGE followed by Western Blotting.
Concentrations of the protein extracts were determined at $A_{280}$ using NanoDrop® ND-1000 UV-Vis Spectrophotometer. Briefly, the spectrophotometer was blanked with 2 $\mu$l Hot Lysis Buffer and 2.0 $\mu$l of each of the protein samples applied and the concentration and purity measured.

2.2.7.6 Sodium Dodecyl Sulphate – Polyacryl Amide Gel electrophoresis: SDS-PAGE.

SDS binds to proteins with a constant ratio of 1.4:1 ($\mu$g/$\mu$g) and thereby confers negative charges to the proteins, which increases proportionally to size and leads to linearization of the protein. Depending on the size of the molecules to be separated, electrophoresis is usually performed using either agarose (large molecules) or polyacrylamide (small molecules) gels. PAGE gels are generated by radical polymerization of acryl amide (AA) and bis–acryl amide (BA) catalysed by radicals set free from ammonium persulphate.

A discontinuous electrophoretic gel/buffer system was employed using the electrophoresis apparatus Novex® Minicell (Invitrogen, Paisley) for SDS–PAGE. Readymade NuPAGE® 10 % Bis–Tris gels of 1.0 mm x 10 wells were used. Protein samples (40 $\mu$g/lane) were mixed 1:4 in NuPAGE® Novex LDS Sample Buffer (4x; Invitrogen, Paisley; containing 4.0 % v/v $\beta$–ME), boiled for 5 mins at 100°C and loaded onto the NuPAGE® 10.0 % Bis–Tris gels. Full Range Rainbow Molecular Weight Marker (Invitrogen, Paisley) was used as a molecular weight marker. The upper buffer electrophoresis chamber was filled with 200 ml of 1x NuPAGE® Novex MOPS SDS Running buffer (20x; Invitrogen, Paisley) and the electrophoresis run at 200 V (constant), 100 mA/gel for 30 mins until the tracking dye reached the bottom of the separating gel.). The gel was washed three times with ddH$_2$O and then incubated for 30 mins in GelCode Blue Stain Reagent to visualize proteins.

2.2.7.7 Western Blot.

Following electrophoresis by SDS–PAGE, proteins were electrotransferred to 0.45 microns Hybond™–C Extra nitrocellulose membrane (Amersham Biosciences UK Ltd., Buck). The electroblotting was performed using 1 x diluted
NuPAGE® Novex Transfer buffer (20x; Invitrogen, Paisley) with added 10.0% v/v CH₃OH in an XCell II™ Blot Module (Invitrogen, Paisley).

Following electrotransfer, protein migration onto the membrane was checked using Ponseau S Staining Solution (0.1% w/v Ponseau S, 5.0 % v/v CH₃COOH). This is a rapid and reversible staining method for locating protein bands on Western blots.

The membrane was then blocked for ≥30 mins at room temperature with 10 ml of 5 % w/v skimmed milk in TBST buffer (block solution) block solution (1% v/v 1.0 M Tris–HCL pH 8.0, 3.0 % 5.0 M NaCl, 0.5% v/v Tween–20 in ddH₂O). Subsequently, the membrane was washed 3 x 5 mins in TBST buffer and incubated for 1.5 h at room temperature (or overnight at 4°C) with specific to the protein of interest primary antibody as detailed below (Table 2.11) in milk–TBST solution. The unbound antibody was then removed by washing the membrane 3 x 5 mins in TBST solution at room temperature. The membrane was then incubated with milk–TBST solution containing secondary antibody (polyclonal goat anti–mouse Immunoglobulins/HRP, polyclonal goat anti–rabbit Immunoglobulins/HRP) at a dilution 1:2000 v/v at room temperature for ≥ 45 mins (Table 11). Following incubation the membrane was washed in TBST for 3 x 5 mins at room temperature.

Proteins were then visualized using a chemiluminescent detection method to detect peroxide labelled secondary antibody using Pierce® ECL Western Blotting Substrate, according to manufacturer instructions. Briefly, Pierce® ECL Western Blotting Substrate (1:1 Pierce® ECL Reagent 1: Pierce® ECL Reagent 2) was directly applied to the membrane and incubated at room temperature for 5 min, after which the membrane was dried and positioned in a film–developing Hypercassette™ (Amersham Pharmacia Biotech, Buckinghamshire, UK). In a dark room, the membrane was exposed to FUJI Medical X–Ray film for 1–2 sec at room temperature. The film was subsequently developed by incubation for 3 mins in KODAK GBX developer/replenisher solution (Sigma–Aldrich, Poole), subsequent washing in ddH₂O and then fixation in KODAK GBX fixer/replenisher solution (Sigma–Aldrich, Poole). Densitometric analysis was performed on the detected bands using image J analysis software.
Table 2.11 Immuno-blotting antibodies: dilutions and conditions.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Conditions</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tubulin</td>
<td>1:2000</td>
<td>1h at RT</td>
<td>Goat anti-mouse HRP-conjugated IgG</td>
<td>1:2000</td>
</tr>
<tr>
<td>MC₁</td>
<td></td>
<td>18 h at 4 °C</td>
<td>Goat anti-rabbit HRP-conjugated IgG</td>
<td></td>
</tr>
<tr>
<td>MC₃</td>
<td></td>
<td>18 h at 4 °C</td>
<td>Goat anti-rabbit HRP-conjugated IgG</td>
<td>1:2000</td>
</tr>
<tr>
<td>HO-1</td>
<td></td>
<td>18 h at 4 °C</td>
<td>Goat anti-rabbit HRP-conjugated IgG</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

In most instances, the membranes were re-probed for the detection of other proteins or secondary proteins such as the standard housekeeping protein α-tubulin. This was achieved by stripping bound antibodies on the nitrocellulose membrane with the commercially available Pierce Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific Inc.) at room temperature for 15 min at 37°C for high affinity antibodies. The membranes were then washed in TBST 3 x 5 min and subsequently blocked in 5 % milk blocking solution for 30 mins at room temperature prior to immuno-blotting. Sufficient removal of antibodies was ensured by incubating the stripped membranes with Pierce® ECL Western Blotting Substrate and consequently exposing them to films.

2.3 Biochemical and analytical methods.

2.3.1 Griess nitrite accumulation assay.

Griess reaction assay (Bartholomew, 1984) is a colorimetric method for determination of iNOS activity in cells following stimulation, it essentially identifies nitrite accumulation within cell culture medium. The assay is based on a chemical reaction utilising sulphanimamide and N–1–napthylethnediamine (NED) under acidic condition (H₂PO₄), whereby nitrite interacts with sulphanimamide in a diazotization reaction forming a diazonium species. Following this the diazonium species binds to NED to form a purple azo compound that can be detected
spectrophotometrically at the dual wavelength of 560 nm and 630 nm, Thereby, provides an indication of nitrite presence in the culture medium.

Cell-free culture supernatants (100 µl) were collected from the experimental plate and transferred to a 96 well plate before an equal volume of Griess reagent (1:1 mixture of 1.0 % w/v sulphanilamide in 5.0 % aqueous H₂PO₄ and 0.1 % w/v aqueous NED) was added to each well. The plate was incubated for 10 min at room temperature for colour development. Following incubation the absorbance of the samples was measured at wavelength of A₅₄₀ nm.

Quantification of NO₂⁻ levels was achieved by comparing the experimental results to a calibration curve. Only standard curves (0 – 1000 µM sodium nitrite in complete medium) with correlation coefficient R² ≥ 0.98 were used to analyze experimental data, as to ascertain that the fitted model explains at least 98 % of the variation in the response variable x (Figure 2.3).

![Figure 2.3 Example of a typical Griess nitrite accumulation assay standard curve. Absorbance values for each sample were measured at 540 nm and nitrite accumulations determined from the standard curve using excel software.](image-url)
2.3.2 Cytokine ELISA.

C-20/A4 and primary chondrocytes were treated at different concentrations with a panel of pro-inflammatory stimuli as describe in section 2.2.6.2. Cell-free supernatants were analysed for pro-inflammatory cytokines (IL–1β, IL–6, IL–8, IL–10 MCP–1). The method of cytokine detection was performed using commercially available ELISA kits (R&D Systems). Firstly, a 100 µl capture antibody specific to the analyte of interest was bound to a 96–well plate overnight in order to create a solid phase. Excess, unbound antibodies were washed off with Wash Buffer and incubated with 300 µl blocking reagent (1% BSA dissolved in PBS) in order to reduce any unspecific binding. The plate was further washed prior to the addition of 100 µl standards, controls and samples were incubated for 2 h to allow the analyte to bind to the capture antibody, any unbound analyte was removed by washing. A detection antibody (100 µl) was then added that binds to a different epitope and incubated for 2 h. Unbound detection antibody was removed by washing and 100 µl Streptavidin–HRP added for 20 mins. The plate was then washed and 100 µl of substrate solution TMB/H2O2 added and incubated for 20 mins. Colour development occurred over this period and was proportional to the amount of bound analyte. The colour development was stopped with the addition of 2N H2SO4 and intensity of the colour measured at A450 nm with wavelength correction. Quantification of cytokine levels was achieved by comparing the experimental results to a standard curve (Figure 2.4).
Figure 2.4 Representative ELISA calibration curves generated using IL-1β (A), IL-6 (B), IL-8 (C), MCP-1 (D) and IL-10 (E).

The standard curves were calculated using a computer-generated 4-PL curve fit. Only standard curves with correlation coefficient $R \geq 0.98$ were used to analyse experimental data, as to ascertain that the fitted model explains at least 98 % of the variation in the response variable $x$. 
2.3.3 Cyclic AMP (cAMP) detection assay.

In functional studies, the alteration of Gi- and Gs-coupled GPCRs is normally examined by detecting the concentration of cAMP. cAMP is the intracellular signalling molecule 3’–5’–cyclic adenosine monophosphate. It modulates intracellular processes by binding to the regulatory units of different protein kinases, thus activating their catalytic subunits. That causes initiation of protein phosphorylation, an event that alters the functionality of the targeted enzymes and/or transcription factors. cAMP is formed by a chemical reaction catalysed by adenylate cyclase, which converts ATP to cAMP and inorganic phosphate and is triggered or inhibited as a result of direct interaction with G-protein α–subunits. Gs and Gi–coupled GPCR activation/inhibition with various agonist or antagonists would therefore result in increase or inhibition of cAMP production, which can be detected using the cAMP assay.

In order to determine if the melanocortin agonists are able to cause cAMP accumulation within chondrocytes, cAMP Biotrak EIA system was used. Human chondrocytic cells C-20/A4 were re–suspended to 1.0 x 10^5 cells/ml and plated in 96–well plates overnight to allow adherence. Medium was then replaced with 1.0 µM IBMX (90.0 µL), and cells then incubated for 30 mins with media, melanocortin peptides or Forskolin (3.0 µM). The media was removed and 100 µl lysis buffer 1B was added to each well, the plate was then placed on a plate shaker for 10 mins to allow for lysis. The lysate standards (0–3200 fmol), substrate blank (B), non–specific c binding (NSB) and samples (100 µl each) were then prepared and added to the plate. Antiserum was added to each well except the NSB and blank wells and incubated at 3 – 5°C for exactly 2 h. Then 50.0 µl cAMP –peroxidase conjugate was added to all wells except the blank and incubated at 3 – 5 °C for 1 h. Plate was then washed with wash buffer and 150 µl of enzyme substrate was added into all wells, and the plate incubated for 30 mins at room temperature. The colorimetric reaction was then stopped by adding 100 µl Stop 1.0 M H_2SO_4 into each well and mixing. The OD was determined at A450

cAMP accumulation levels were then calculated by comparing the experimental results to a standard curve (Figure 2.5).
2.3.4 MTT cell proliferation assay.

MTT cell proliferation assay measures mitochondrial function as an indication of cell viability (Mosmann, 1983). The assay is often used to determine the cytotoxicity of potential medicinal agents and toxic materials, since those would stimulate or inhibit cell viability and growth. The principle of this assay is to assess the ability of mitochondrial succinate dehydrogenase for metabolizing the yellow soluble form of MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, a yellow tetrazole) by reducing it to purple insoluble formazan in living cells (Figure 2.6) (Mosmann, 1983). The amount of formazan produced is directly proportional to the number of active cells and thus can be measured spectrophotometrically to quantify the formazan product.

Briefly, chondrocytes were treated with or without pro–inflammatory stimuli ± a range of melanocortin peptides/ anti–inflammatory drugs for 1 – 72h, supernatant collected and removed. Fresh media containing MTT Stock Solution (5.0 μg/ml;
10X) was added to each well and cells incubated for 2 h at 37°C. MTT solution was then discarded and the resulting formazan deposits were dissolved in DMSO for 15 min prior to absorbance measurement at $A_{570}$ and $A_{630}$. Samples containing only DMEM + C-20/A4 chondrocytes represented 100% viability (positive control), whereas 0% was represented from DMEM without cells (negative control).

Figure 2.6 General reaction scheme for the reduction of MTT to formazan by mitochondrial succinate dehydrogenase (Mosmann, 1983).
2.3.5 Caspase-Glo 3/7 apoptosis assay.

The Caspase-Glo® 3/7 assay is a homogenous luminescent assay that measures caspase-3 and caspase-7 activities. These caspases belong to a family of cysteine/aspartic acid-specific proteases, which plays key effector roles in mammalian cells apoptosis (Nicholson and Thornberry, 1997, Thornberry and Lazebnik, 1998, Garcia-Calvo et al., 1999).

In order to detect cell apoptosis following various treatments of the cells, C-20/A4 cells were plated at 2.0 x 10^4/well in 96-well plates with 100 μl of DMEM medium supplemented with 1 % Pen/Strep and incubated for 24 h to allow adherence. Cells were treated with various agents (pro-inflammatory stimuli ± relevant drug) for 6 h and caspase 3 and 7 activity was then evaluated according to the manufacturer’s protocol (Promega).

100 μl of Caspase-Glo 3/7 Reagent was added negative control cells or treated cells in DMEM medium. The blank reaction was used to measure background luminescence associated with the C20/A4 cell culture system and the Caspace-Glo reagent. The negative control reactions were prepared in order to detect and record basal levels of caspase activity in the C-20/A4 cell culture system.

Content was mixed gently on a plate shaker at 300-500 rpm for 30 sec and left at room temperature to incubate for 1 h, following which luminescence was measured using plate-reading luminometer.

2.4 Data analysis and statistics.

For both in vitro and in situ studies, experiments were performed in triplicates unless stated otherwise in the individual figure legends. Bovine articular chondrocytes were extracted and pooled together from 3 – 4 animal joints per experiment (N=3/4) and performed also in triplicate. Data are reported as mean ± standard error of the mean (SEM) unless otherwise stated. Significant differences were determined by One-way/Two-way analysis of variance (ANOVA), followed by Dunnet’s post-test, or Bonferroni’s multiple comparison tests as appropriate using GraphPad Prism 5.0, (GraphPad Software, CA, USA). Values of p≤ 0.05 were considered significant, where p≤ 0.05 (*), p≤ 0.01 (**) and p≤ 0.001 (***).
Chapter 3
Results
3.1 Effect of pro-inflammatory stimuli on C-20/A4 chondrocyte function.

It is increasingly appreciated that mediators typically associated with inflammatory arthritis, such as catabolic cytokines and nitric oxide are produced by chondrocytes in OA. The role these mediators play in the progression of cartilage degradation during OA is an area of intense investigation.

In this thesis, I have evaluated whether human C-20/A4 chondrocytes might be used as a surrogate model for evaluating the effect of anti-inflammatory drugs including the effect of melanocortin peptides on inflammatory mediator release from activated chondrocytes. I have investigated the cells response to inflammatory stimuli (TNF-α, LPS, Zymosan, H₂O₂ and MSU crystals) on synthesizing and releasing pro-inflammatory mediators, such as nitric oxide and various catabolic cytokines and chemokines including IL-1β, IL-6, IL-8 and MCP-1.

3.1.1 Effect of TNF-α, LPS, H₂O₂ and MSU crystals on nitrite concentration and cell viability.

Initially, it was investigated whether a panel of stimuli (TNF-α, LPS, Zymosan, H₂O₂ and MSU crystals) were able to cause activation of the iNOS gene and this was determined by measuring the release of nitrate from stimulated chondrocytes. Nitric oxide is an important signalling molecule that acts in many tissues to regulate a diverse range of physiological processes. Cytotoxicity as a result of a substantial NO-formation is an established apoptosis initiating factor (Brune et al., 1999). Following stimulation, chondrocyte viability was assessed by MTT assay to ascertain the effect of the inflammatory stimuli on this parameter.

C-20/A4 chondrocytes were plated in monolayer in DMEM media (50.0 U/ml Penicillin, 50.0 µg/ml Streptomycin) for 24 h prior to stimulation with TNF-α (20.0 – 80.0 pg/ml), LPS (0.1 – 10.0 µg/ml), H₂O₂ (0.01 – 1.0 %) or MSU crystals (0.1 – 1.0 µg/ml) for 6 h. Cell free supernatants were collected and analysed for nitrite release by Griess assay. In unstimulated cells there was a basal release of nitrite (1.227 ± 1.214 µM) and the effect of the inflammogens were compared to this
value. In initial experiments C-20/A4 chondrocytes were stimulated with high concentrations of \( \text{H}_2\text{O}_2 \) to determine whether C-20/A4 chondrocytes produce nitrite compared to unstimulated control cultures.

\( \text{H}_2\text{O}_2 \) caused a concentration-dependent increase in NO release, where 0.01\%, 0.1\% and 1.0\% \( \text{H}_2\text{O}_2 \) led to 139.28 ± 6.24 \( \mu \text{M} \), 175.67 ± 7.18 \( \mu \text{M} \) and 210.12 ± 10.31 \( \mu \text{M} \) respectively (\( p \leq 0.001 \)), thereby indicating that the chondrocytes can release substantial quantities of nitrite when stimulated (Figure 3.1 A). After validating the ability of C-20/A4 cells to respond to oxidative stress by activating iNOS, we tested the response of the chondrocytes to TNF-\( \alpha \), LPS and MSU crystals stimulation.

Figure 3.1 B demonstrates the effect of TNF-\( \alpha \) (20.0 – 80.0 pg/ml) on nitrite formation. Stimulation of the human cell-line chondrocytes led to a significant release of nitrite, whereby TNF-\( \alpha \) (20.0 pg/ml) caused 19.18 ± 6.79 \( \mu \text{M} \), which was not substantially altered upon increasing the concentrations, however, maximal release was observed at 80.0 pg/ml (20.86 ± 6.33 \( \mu \text{M} \), \( p \leq 0.05 \) vs. control). Next, it was evaluated whether the observed effect was due to the particular stimulus being used; therefore nitrite release in the presence of LPS (0.1 – 10.0 \( \mu \text{g/ml} \)) was examined (Figure 3.1) and significant alterations were detected: 36.35 ± 7.95 \( \mu \text{M} \), 30.35 ± 9.64 mM and 24.99 ± 7.21 \( \mu \text{M} \) for 1.0, 3.0 and 10.0 \( \mu \text{g/ml} \) LPS respectively (\( p \leq 0.05 \) for all concentrations tested). Conversely, MSU crystals (30 – 1000 ng/ml) did not cause a significant increase in nitrite release compared to unstimulated controls (\( p > 0.05 \); Figure 3.1 D).

Following identification of C-20/A4 chondrocytes capability to produce NO, cell viability was assessed by MTT assay in the presence of these stimuli, thereby confirming the cytotoxicity of the given stimulus in this cell-line model. To ensure that cell death could be measured in C-20/A4 chondrocytes, cells were stimulated with 0.01 \%, 0.1 \% and 1.0 \% \( \text{H}_2\text{O}_2 \), which caused a concentration-dependent decrease in cell viability with 82.25 ± 4.66 \%, 87.14 ± 4.23 \% and 92.80 ± 1.29 \% cell death respectively (\( p < 0.001 \); Figure 3.2A). Similarly, TNF-\( \alpha \) (20.0 – 80.0 pg/ml) caused a significant concentration-dependent decline in C-20/A4 chondrocyte viability (Figure 3.2 B), where 20.0 pg/ml caused 16.14 \% ±
4.09 % reduction in viability (p≤ 0.05). Analogous effects were noted following 40.0 pg/ml (11.8 ± 3.4%, n.s.), whilst at higher concentrations (60.0 pg/ml) there was 27.58 ± 2.85% (p≤ 0.01) rate of cell death, which was slightly increased at 80.0 pg/ml to 30.22 ± 1.18 % (p≤ 0.001) compared to untreated cells (Figure 3.2 B). C-20/A4 chondrocytes were then stimulated with LPS, which caused a 23.29 ± 3.14 % (p≤ 0.001), 19.24 ± 2.83 % (p≤ 0.001) and 17.42 ± 3.23 % (p≤ 0.01) cell death at 1.0, 3.0, and 10.0 µg/ml concentrations respectively.

MSU crystals (30 – 1000 ng/ml) did not alter cell viability of C-20/A4 chondrocytes compared to unstimulated cells. This finding, combined with the observation that it did not induce nitrite release led to discontinuation of further use of this inflammogen (Figure 3.1 D, Figure 3.2 D).
Figure 3.1. The effect of inflammatory stimuli on nitric oxide production from C-20/A4 chondrocytes.

C-20/A4 chondrocytes (1.5 x 10^5 cells/cm²) were plated in serum-free DMEM and stimulated with H_2O_2 (0.01 – 1.0 %; Panel A), TNF-α (20.0 – 80.0 pg/ml; Panel B), LPS (1.0 – 10.0 µg/ml; Panel C) or MSU crystals (0.1 – 1.0 µg/ml; Panel D) for 6 h. Cell-free supernatants were then collected and analysed for nitrite release via Griess assay. Dotted line represents basal release of nitrite (1.23 ± 1.21 µM). Data are presented as Mean ± SEM of n=9 experiments, assessed in triplicate. *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001 vs. untreated control cultures.
Figure 3.2. The effect of inflammatory stimuli on C-20/A4 chondrocytes viability.

C-20/A4 chondrocytes ($1.5 \times 10^5$ cells/cm$^2$) were plated in serum-free DMEM (280 and stimulated with H$_2$O$_2$ (0.01 – 1.0 %; Panel A), TNF-α (20.0 – 80.0 pg/ml; Panel B), LPS (1.0 – 10.0 µg/ml; Panel C) or MSU crystals (0.1 – 1.0 µg/ml; Panel D) for 6 h. Cell viability was then determined via MTT reduction assay. Dotted line represents control sample cell viability – untreated cells (100%). Data are presented as Mean ± SEM of n=9 experiments, assessed in triplicate. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ vs. untreated control cultures.
3.1.2 Detection of pro-inflammatory cytokines release following stimulation of C-20/A4 chondrocytes with TNF-α and LPS.

The importance of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 IL-8 in the induction of catabolic processes in chondrocytes has been recognized (Pelletier et al., 1991, Shinmei et al., 1991, Lotz et al., 1992, Reboul et al., 1996, Melchiorri et al., 1998, Shlopov et al., 2000, Fernandes et al., 2002, Schuerwegh et al., 2003, Rai et al., 2008, Goldring et al., 2008). The major source of pro-inflammatory cytokines in OA has been thought to be activated synovium or infiltrating inflammatory cells, however strong evidence exists that chondrocytes can also release these mediators and drive the inflammatory response within the joint (Goldring, 2000; Goldring et al., 2011). In order to investigate whether human C-20/A4 chondrocytes are capable of releasing pro-inflammatory cytokines, cells were stimulated with TNF-α and LPS and mRNA and protein levels of pro-inflammatory cytokines were determined.

3.1.2.1 Effect of TNF-α and LPS on IL6 and IL8 mRNA expression in C20/A4 chondrocytes.

In initial experiments, the effect of TNF-α (60.0 pg/ml) and LPS (1.0 µg/ml) was investigated on IL6 and IL8 mRNA expression levels following 0 – 48 h incubation periods. Total RNA was extracted from stimulated and non-stimulated cells and conventional RT-PCR amplification reactions with specific primers for IL6 and IL8 genes (Table 2.9) were used. Gene expression was visualized on 2 % agarose gels, run in conjunction with β-actin (Figure 3.5 A). RT-PCR showed that C-20/A4 chondrocyte respond to TNF-α (60.0 pg/ml), a concentration chosen from previous experiments to cause a sub-maximal rate of cell death and nitrite release, thereby substantially increasing IL6 and IL8 expression in time-dependent manner (Figure 3.3 A). Maximal mRNA levels of IL6 and IL8 were detected 2 h post-stimulation gradually decreased in the period between 6 – 48 h, as determined by densitometric analysis (Figure 3.3 B and C). Incubation period of 6 h was chosen as suitable for subsequent experiments as it led to sub-maximal release of IL6 and IL8, thereby allowing for the concentration dependent effect of TNF-α to be investigated. TNF-α (20.0 – 80.0 pg/ml) caused increases
in \textit{IL6} and \textit{IL8} with densitometric quantification showing a 53-fold increase in expression of \textit{IL6} peaking at 40.0 pg/ml TNF-\textalpha{} (Figure 3.4 A), and a 9-fold increase in \textit{IL8} compared to control (Figure 3.4 B and C).

In order to clarify the response of C-20/A4 chondrocytes to LPS, the effect of various concentrations on pro-inflammatory cytokine expression was investigated. LPS induced a concentration-dependent increase in the expression of \textit{IL6} and \textit{IL8} (Figure 3.4 C and D), whereby chondrocytes were notably activated following concentrations as low as 0.1 \mu{}g/ml, and reaching a 72-fold increase in \textit{IL6} and 13.5-fold increase in \textit{IL8} expression at 1.0 \mu{}g/ml compared to untreated controls ($p \leq 0.001$). Each value was normalized to the respective \textit{\beta{}-actin} expression. Shown are the means of four independent experiments.
Figure 3.3. Densitometric quantification of IL6 and IL8 expression in C-20/A4 chondrocytes treated with TNF-α (60.0 pg/ml) for 0-48 h.

C-20/A4 chondrocytes were stimulated with TNF-α (60.0 pg/ml) at time 0 and total RNA extracted 0-48 h post-stimulation. PCR amplification with the respective primers for IL6 and IL8 was used to detect and quantify gene expression on 2% agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified IL6 (Panel B) and IL8 (Panel C) shown in arbitrary units, each value normalized to the respective β-actin expression. Data are presented as Mean ± SEM of four independent experiments *p≤0.05, **p≤0.01, ***p≤ 0.001).
Figure 3.4. Densitometric quantification of IL6 and IL8 expression in C-20/A4 cells treated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 µg/ml) for 6 h.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 µg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for IL6 and IL8 was used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified IL6 (Panel B and C) and IL8 (Panel D and E) shown in arbitrary units, each value normalized to the respective β-actin expression. Data are presented as Mean ± SEM of four independent experiments *\(p \leq 0.05\), **\(p \leq 0.01\), ***\(p \leq 0.001\).
3.1.2.2 Effect of TNF-α and LPS on IL-6 and IL-8 cytokine release from C20/A4 chondrocytes.

Following identification of TNF-α and LPS ability to induce IL6 and IL8 expression in C-20/A4 chondrocytes, the effect of various concentrations of these stimuli over a time course was investigated, thereby confirming the genuine translation of IL6 and IL8 mRNA to protein. C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) and LPS (1.0 – 10.0 µg/ml) for 0, 2, 6 and 24 h and cell-free supernatants were collected and analysed. Stimulation for 2 h with TNF-α (20.0 – 80.0 pg/ml) resulted in well-defined bell-shaped curve effect in IL-6 release, peaking at 60.0 pg/ml (99.23 ± 13.81 pg/ml; p ≤ 0.05) and decreasing thereafter with 80.0 pg/ml leading to 74.55 ± 17.89 pg/ml (p ≤ 0.05; Figure 3.5 A). Lower concentrations of TNF-α failed to illicit significant IL-6 release at this time-point as compared to control (DMEM treated cultures). Subsequently, a longer, 6 h incubation period was employed to investigate IL-6 production from chondrocytes in response to TNF-α, with 20.0 pg/ml and 40.0 pg/ml of TNF-α caused 45.52 ± 8.3 pg/ml and 59.64 ± 2.4 pg/ml of IL-6 respectively (p ≤ 0.05; Figure 3.5 B). As previously seen, maximal release following 2 h of stimulation was caused by 60.0 pg/ml of TNF-α with 117.1 ± 26.09 pg/ml (p ≤ 0.05), which slightly decreased following treatment with 80.0 pg/ml TNF-α (84.31 ± 5.69 pg/ml; p ≤ 0.05). Analogous effects were observed at 24 h, when a bell-shaped release of IL-6 was detected with 20.0 pg/ml and 40.0 pg/ml causing 52.79 ± 2.60 pg/ml and 63.30 ± 6.97 pg/ml of IL-6 respectively (p ≤ 0.05). Increasing the concentrations led to the detection of a maximal release of 154.30 ± 10.32 pg/ml (p ≤ 0.01) detected at 60.0 pg/ml, which decreased at 80.0 pg/ml TNF-α (139.34 ± 11.48 pg/ml (p ≤ 0.01; Figure 3.5 C).

The effect of LPS (0.1 – 10.0 µg/ml) was subsequently determined and confirmed to cause a concentration-dependent increase in IL-6 at 2 h compared to untreated control cultures (Figure 3.5 D). Concentrations lower than 1.0 µg/ml did not cause statistically significant difference compared to control values (p ≤ 0.05), but higher concentrations of LPS led to 73.467± 13.83 pg/ml, 176.85 ± 23.5 pg/ml and 152.34 ± 6.44 pg/ml of IL-6 production (p ≤ 0.05) for 1.0, 3.0 and 10.0 µg/ml respectively. Similar observations were made when C-20/A4 chondrocytes were
treated with LPS for 6 h; however, here, in accordance with RT-PCR detected levels, 0.1 \( \mu \text{g/ml} \) and 0.3 \( \mu \text{g/ml} \) of LPS caused a significant increase in IL-6 production, leading to 61.33 ± 7.11 pg/ml and 68.67 ± 10.33 pg/ml respectively (\( p \leq 0.05 \); Figure 3.5 E). At 24 h post-stimulation LPS (0.1 – 10.0 \( \mu \text{g/ml} \)), a maximal release of 134.93 ± 14.72 pg/ml of IL-6 was recorded following stimulation with LPS (3.0 \( \mu \text{g/ml} \); \( p \leq 0.01 \); Figure 3.5 F) There was no significant difference between the potency of the various concentrations probably due to a plateau, which is reached at concentrations this high.

Consequently the translation of IL-8 mRNA to protein was determined. C-20/A4 chondrocytes were stimulated with TNF-\( \alpha \) and LPS for 0, 2, 6 and 24 h and a bell-shaped response was recorded regardless of the time-point. The results, reported in Figure 3.6 A, demonstrated the lack of efficacy of low concentrations of TNF-\( \alpha \) (20.0 and 40.0 pg/ml) at short incubation periods (2 h) in the chondrocyte system. However, 2 h-stimulation with 60.0 pg/ml and 80.0 pg/ml of TNF-\( \alpha \) caused significant IL-8 production with 130.23 ± 9.5 pg/ml and 142.65 ± 12.5 pg/ml respectively (\( p \leq 0.05 \)), (Figure 3.6 A). These amounts were compared to basal levels of IL-8 synthesis (63.34 ± 5.32 pg/ml). TNF-\( \alpha \) caused a bell-shaped response in IL-8 production at 6 h post-simulation, peaking at 60.0 pg/ml (205.90 ± 27.1 pg/ml; \( p \leq 0.01 \)), and slowly decreasing following stimulation with 80.0 pg/ml of TNF-\( \alpha \) (145.68 ± 4.72 pg/ml; \( p \leq 0.01 \); Figure 3.6B). In contrast to the effect at 20.0 and 40.0 pg/ml of TNF-\( \alpha \) at 2h, these concentrations led to significant increase in IL-8 production following 6 h incubation (120.38 ± 3.67 pg/ml and 131.8.41 pg/ml; \( p \leq 0.05 \); Figure 3.6 B) compared to control (72.65 ± 7.34 pg/ml). Lastly, TNF-\( \alpha \) effect at 24 h was determined with 20.0 and 40.0 pg/m leading to 348.57 ± 53.23 pg/ml and 391.36 ± 35.7 pg/ml of IL-8 respectively (\( p \leq 0.01 \)), compared to control (79.33 ± 15.65 pg/ml).

LPS (0.1 – 10.0 \( \mu \text{g/ml} \)), being a bacterial lipopolysaccharide has been shown to cause joint and cartilage inflammation (Lotz et al., 1992). At all concentrations and time-points tested it caused a significant increase in IL-8 production. At 2 h, 6 h and 24 h there was a concentration-dependent increase leading to a maximal release of IL-8 at 3.0 \( \mu \text{g/ml} \) with 189.5 ± 25.46 pg/ml, (\( p \leq 0.05 \)), 243.53 ± 18.31 pg/ml (\( p \leq 0.01 \)) and 272.54 ± 10.29 pg/ml (\( p \leq 0.01 \)) respectively. All other
concentrations caused similarly significant amounts of IL-8, but were not different from one another within the incubation period (Figure 3.6 D-F).

Figure 3.5 Effect of pro-inflammatory stimuli on IL-6 release from C20/A4 chondrocytes.
C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml; Panel A – C) or LPS (0.1 – 10.0 µg/ml; Panel D – F) and cell-free supernatants collected at 0, 2, 6 and 24 h. IL-6 release was then determined by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, \(^* \text{p} \leq 0.05\), \(^{**} \text{p} \leq 0.01\) vs. Time 0 (dotted line).
Figure 3.6. Effect of pro-inflammatory stimuli on IL-8 release from C20/A4 chondrocytes.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml; Panel A – C) or LPS (0.1 – 10.0 µg/ml; Panel D – F) and cell-free supernatants collected at 0, 2, 6 and 24 h. IL-8 production was then determined by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤0.05, **p≤0.01 vs. Time 0 (dotted line).
3.1.2.3 Effect of TNF-α and LPS on IL-1β and MCP-1 cytokine release from C-20/A4 chondrocytes.

In separate experiments C-20/A4 chondrocytes were stimulated with different concentrations of TNF-α (20.0 – 80.0 pg/ml) and LPS (1.0 – 10.0 µg/ml) for 0, 2, 6 and 24 h and cell-free supernatants were analysed for IL-1β and MCP-1 by ELISA. Given that IL-1β is considered to be active locally within cartilage and leads to matrix destruction, IL-1β levels were measured (Attur et al., 2000; Fernandes et al., 2002; Kapoor et al., 2011). In addition, to assess chondrocyte contribution to the chemotactic environment of inflamed joints the intracellular content of the CC (IL-1β) and CXC chemokine (MCP-1) was investigated, with specific interest in MCP-1 production (Villager et al., 1992; Borzi et al., 1999). TNF-α and LPS caused a significant increase in IL-1β release (p ≤ 0.05) compared to unstimulated control cells. Treatment with TNF-α caused a significant concentration-dependent increase in IL-1β at 2, 6 and 24 h post-stimulation.

Following 2 h stimulation, the lower concentrations (20.0 and 40.0 pg/ml) of TNF-α didn’t cause significant up-regulation in IL-1β production, consistent with the results obtained for IL-6 and IL-8. The maximal release of 16.74 pg/ml at this time point was induced by 80.0 pg/ml (p ≤ 0.05; Figure 3.7 A), and these observations were confirmed at 6 h, where 80.0 pg/ml caused 28.35 ± 3.25 pg/ml IL-1β to be released (p ≤ 0.05; Figure 3.7 B). However, at 6 h, 40.0 pg/ml and 60.0 pg/ml of TNF-α also led to significant increase in the concentration of IL-1β, 15.73 ± 1.1 pg/ml and 24.37 ± 8.69 pg/ml respectively (p ≤ 0.05). IL-1β was not detectable at 24 h following stimulation with 80.0 pg/ml, however at 60.0 pg/ml, a small but detectable amount of IL-1β was observed (14.61 ± 3.1 pg/ml, p ≤ 0.05; Figure 3.7 C).

LPS (3.0 µg/ml) stimulation of C-20/A4 chondrocytes for 2 h caused the maximal release of IL-1β for this time point with 17.23 ± 2.86 pg/ml (p ≤ 0.05; Figure 3.7 D). Following 6 h stimulation, LPS caused a concentration-dependent bell-shaped response, with 1.0 µg/ml causing 26.55 ± 10.31 pg/ml of IL-1β to be released (p ≤ 0.05; Figure 3.7 E). At 24 h post-stimulation, a plateau was reached,
with all concentrations causing similar amounts of IL-1β to be released ($p \leq 0.05$; Figure 3.7 F).

Next, TNF-α (20.0 – 80.0 pg/ml) effect was evaluated on MCP-1 production, where stimulation for 2 h (Figure 3.8 A) did not yield statistically significant increases in MCP-1 compared to basal levels (46.18 ± 14.21 pg/ml; $p \leq 0.05$). TNF-α (20.0 pg/ml) did not cause any significant change in MCP-1 levels regardless of the incubation periods ($p > 0.05$) evaluated, however, with increasing concentrations of the cytokine at 6 h (Figure 3.8 B), there was a significant increase in MCP-1 production of 69.39 ± 4.21 pg/ml ($p \leq 0.05$), 101.2 ± 16.37 pg/ml ($p \leq 0.05$) and 119.92 ± 8.74 pg/ml ($p \leq 0.05$) for TNF-α 40.0 pg/ml, 60.0 pg/ml and 80.0 pg/ml respectively. MCP-1 levels at 24 h following stimulation with TNF-α (40.0 – 80.0 pg/ml; Figure 3.8 C) followed a bell-shaped curve, where 40.0, 60.0 and 80.0 pg/ml TNF-α causing 70.43 ± 5.23 pg/ml ($p \leq 0.05$), 101.34 ± 12.09 ($p \leq 0.05$) and 93.32 ± 6.42 pg/ml ($p \leq 0.05$) respectively compared to basal production (39.29 ± 11.28 pg/ml).

Subsequently, LPS was evaluated and found to stimulate significant rise in MCP-1 release with a maximal response achieved at 3.0 and 10.0 µg/ml following 6 h incubation (Figure 3.8 E), with 68.34 ± 4.52 pg/ml and 70.11 ± 3.32 pg/ml respectively compared to untreated controls (46.18 ± 14.21 pg/ml). At 2 h post-stimulation (Figure 3.8 D), MCP-1 release was lower 25.29 ± 2.38 pg/ml (0.3 µg/ml; $p \leq 0.05$), 41.34 ± 12.23 pg/ml (1.0 µg/ml; $p \leq 0.05$), 23.55 ± 6.89 pg/ml (3.0 µg/ml, $p \leq 0.05$) and 32.43 ± 12.48 pg/ml (10.0 µg/ml; $p \leq 0.05$). Incubation periods longer than 6 h did not induce statistically significant increases in MCP-1 release from C-20/A4 chondrocytes compared to basal levels (39.31 ± 8.89; $p \leq 0.05$; Figure 3.8 F).
Figure 3.7. Effect of pro-inflammatory stimuli on IL-1β release from C20/A4 chondrocytes.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml; Panels A – C) or LPS (0.1 – 10.0 µg/ml; Panels D – F) and cell-free supernatants collected at 0, 2, 6 and 24 h and IL-1β determined by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05 vs. Time 0 (Dotted line).
Figure 3.8. Effect of pro-inflammatory stimuli on MCP-1 release from C20/A4 chondrocytes.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml; Panel A – C) or LPS (0.1 – 10.0 µg/ml; Panel D – F) and cell-free supernatants collected at 0, 2, 6 and 24 h and analysed for MCP-1 by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p ≤ 0.05 vs. Time 0 (Dotted line).
3.1.2.4 Time dependent release of pro-inflammatory cytokines following C-20/A4 chondrocyte stimulation.

In order to fully understand the response of C-20/A4 chondrocytes to inflammmogens, they were stimulated with TNF-α at 60.0 pg/ml and LPS at 1.0 µg/ml (the most potent concentrations identified from previous experiments) for a longer time course investigation including incubation periods of up to 72 h. Cell-free supernatants were collected at each individual time point and subsequently analysed for IL-6 and IL-8 release by ELISA.

Cultured C-20/A4 cells had a basal release of IL-6 (21.09 ± 18.49 pg/ml) and IL-8 (72.65 ± 13.32 pg/ml). TNF-α (60.0 pg/ml) and LPS (1.0 µg/ml) caused a significant time-dependent increase in IL-6 (Figure 3.9 A, B) and IL-8 (Figure 3.9 C, D), with maximal production detected at 48 h post-incubation, with 154.30 ± 10.32 pg/ml and 159.65 ± 20.86 pg/ml (p≤ 0.001) of IL-6 following TNF-α and LPS stimulation respectively. Maximal release of IL-8 was detected at 48 h (558.90 ± 11.34 pg/ml, p≤ 0.001), whilst following LPS stimulation it was detected at 72 h (378.79 ± 46.86 pg/ml, p≤ 0.001). Release of these pro-inflammatory cytokines following 2 h of incubation with TNF-α (60.0 pg/ml) and LPS (1.0 µg/ml) caused significant increases of 99.23 ± 13.81 pg/ml (p≤ 0.05) and 73.47 ± 13.82 (p≤ 0.05) of IL-6 and 130.23 ± 9.50 pg/ml (p≤ 0.05) and 172.64 ± 14.97 pg/ml (p≤ 0.01) IL-8 respectively. At later time-points there was an increase in these cytokines, such that following 4 h stimulation with LPS there was a marked up-regulation of IL-6 and IL-8 with 112.0 ± 11.3 pg/ml (p≤ 0.05) and 219.45 ± 12.22 pg/ml for IL-6 and IL-8 respectively. Incubation of chondrocytes with TNF-α (60.0 pg/ml) and LPS (1.0 µg/ml) for 6 h caused 117.1 ± 26.1 pg/ml and 135.99 ± 20.13 pg/ml release of IL-6, for the two inflammogens respectively, and 205.92 ± 27.10 pg/ml and 223.74 ± 15.33 pg/ml release of IL-8, respectively (p≤ 0.05).
Figure 3.9. Effect of TNF-α and LPS on IL-6 and IL-8 release in C-20/A4 cells over 0-72 h period.

C-20/A4 chondrocytes were stimulated with TNF-α (60.0 pg/ml; dark blue bars) or LPS (1.0 µg/ml; light blue bars) and cell-free supernatants collected at 0 – 72 h. IL-6 (Panel A, B) and IL-8 (Panel C, D) were then determined by ELISA. Data are presented as Mean ± SEM of n=7 independent experiments repeated in triplicate, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 vs. Time 0 (represented by a dotted line).
3.1.3 Effect of TNF-\( \alpha \) and LPS on \textit{MMP1}, \textit{MMP3} and \textit{MMP13} expression in C20/A4 chondrocytes.

OA is increasingly characterised by release of pro-inflammatory cytokines and other mediators of the host inflammatory response and cartilage degradation initiated by MMPs. Generally, collagenase expression induced by TNF-\( \alpha \) is thought to be NF\( \kappa \)B-dependent, a pathway also used by TLRs (Zhang et al., 2008). Initially, the effects of TNF-\( \alpha \) and LPS (TLR4 ligand) were investigated on \textit{MMP1}, \textit{MMP3} and \textit{MMP13} expression by C20/A4 chondrocytes. The effect of TNF-\( \alpha \) (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 \( \mu \)g/ml) on MMPs levels were at first evaluated following 6 h stimulation of C-20/A4 chondrocytes and the total RNA extracted from stimulated and non-stimulated cells was analysed for transcription of these genes by RT-PCR.

LPS and TNF-\( \alpha \) caused increases in \textit{MMP1} (Figure 3.10 A) and \textit{MMP13} (Figure 3.11 A) and decreased expression of \textit{MMP3} in C-20/A4 cells at 6 h. (Figure 3.12 A). TNF-\( \alpha \) (20.0 – 80.0 pg/ml) caused significant increases in \textit{MMP1} expression (Figure 3.10 B), with 20.0 pg/ml causing 1.5-fold increase compared to basal (0.21 ± 0.02 au to 0.33 ± 0.04 au, \( p \leq 0.05 \)). Increased concentrations of TNF-\( \alpha \) (40.0 and 60.0 pg/ml) led to a plateau effect of 8-fold up-regulation of \textit{MMP1} with expression levels of 1.66 ± 0.22 au and 1.71 ± 0.11 au respectively (\( p \leq 0.01 \) for both concentrations), whilst 80.0 pg/ml of TNF-\( \alpha \) caused a slightly less profound but similar increase to 1.52 ± 0.23 au representing 7-fold increase in expression (\( p \leq 0.01 \)). LPS stimulation caused a bell-shaped response with a significant increase in \textit{MMP1} expression at 0.1 \( \mu \)g/ml (8-fold increase; 1.78 ± 0.21 au, \( p < 0.01 \)) and at 1.0 \( \mu \)g/ml (7-fold increase; 1.47 ± 0.1 au, \( p \leq 0.01 \)), with a maximal 12-fold up-regulation detected following stimulation with 0.3 \( \mu \)g/ml LPS (2.18 ± 0.3 au, \( p \leq 0.01 \); Figure 3.10 C).

Densitometric analysis of \textit{MMP13} expression showed a concentration dependent increase in expression (Figure 3.11 A), whilst no detection was observed in non-stimulated C-20/A4 chondrocytes. TNF-\( \alpha \) (20.0 and 40.0 pg/ml) caused an increase in \textit{MMP13} expression to 0.057 ± 0.03 au and 0.1 ± 0.02 au, respectively (\( p \leq 0.05 \)). TNF-\( \alpha \) 60.0 pg/ml and 80.0 pg/ml caused 0.23 ± 0.01 ± 0.002 au and
0.34 ± 0.07 au respectively (p≤ 0.001; Figure 3.11 B). Similarly, LPS (0.1 – 1.0 µg/ml) at all concentrations led to significant increases in MMP13 expression levels (Figure 3.11 C), with 0.1 µg/ml causing 0.13 ± 0.003 au and higher concentrations causing 0.205 ± 0.002 au and 0.2 ± 0.03 au (p≤ 0.001 both) expression for 0.3 µg/ml and 1.0 µg/ml respectively.

MMP3 was then evaluated as it has been shown to be highly expressed in healthy hip cartilage, and significantly reduced in osteoarthritic hip cartilage (Kevorkian et al., 2004). Both TNF-α and LPS significantly reduced MMP3 levels (Figure 3.12 A). TNF-α stimulation caused a bell-shaped inhibition in MMP3 expression with 60.0 pg/ml, with a 46% down-regulation of the protease expression from 0.580 ± 0.051 au to 0.31 ± 0.05 au (p≤ 0.05; Figure 3.12 B). Treatment with LPS caused a concentration dependent decrease in MMP3 expression with 1.0 µg/ml causing a complete inhibition in expression compared to untreated controls (p≤ 0.001). Lower concentrations of LPS caused significant inhibition with 0.1 µg/ml and 0.3 µg/ml leading to 64% and 77% down-regulation of MMP3 expression (0.21 ± 0.04 and 0.13 ± 0.06 au, p≤ 0.01) compared to control cultures (Figure 3.12 C).

Following identification of MMP1, MMP3 and MMP13 mRNA expression at 6 h, the effect of TNF-α in a time-dependent manner was investigated. C-20/A4 chondrocytes were stimulated with TNF-α (60.0 pg/ml) for 0 – 48 h. MMP1 was markedly increased in a time-dependent manner (Figure 3.13 A). Unlike IL6 and IL8 expression, which peaked at 2 h post-stimulation, TNF-α stimulation of MMP1 did not cause significant change in expression at this time point (0.31 ± 0.04 au compared to untreated 0.22 ± 0.03 au). However, at 6 h there was a marked 8-fold increase to 1.71 ± 0.11 au (p<0.001). The expression of MMP1 continued to increase over the time course to 2.12 ± 0.22 au (p≤ 0.001) at 24 h and was reduced at 48 h (1.72 ± 0.23 au, 8-fold; p≤ 0.001; Figure 3.13 B).

Similarly, MMP13 expression was unchanged following 2 h stimulation with TNF-α (0.021 ± 0.022 au; p>0.05); however, expression was significantly increased at 6 h with a 11.5-fold increase in expression compared to 2 h (p<0.001; Figure 3.13 D). Increasing the length of the incubation time caused further increases in
expression of MMP13 with 0.29 ± 0.03 au and 0.34 ± 0.07 au at 24 h and 48 h post-incubation respectively.

Treatment of C-20/A4 with TNF-α (60.0 pg/ml) caused a significant reduction of MMP3. It was significantly reduced by 55% down to 0.26 ± 0.05 au (p ≤ 0.01) at 2 h, by 36% down to 0.37 ± 0.42 au (p ≤ 0.05) and at 24 h the expression of MMP3 was down-regulated to 0.31 ± 0.05 au, a 46% decrease (p ≤ 0.05) as compared to untreated controls (0.580 ± 0.051 au). At 48 h post stimulation, there was a slight non-significant inhibition of MMP3 expression (Figure 3.13 C).
Figure 3.10. Densitometric quantification of *MMP1* expression in C-20/A4 cells treated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 µg/ml) for 6 h.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) or LPS (0.1 – 1.0 µg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *MMP1* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *MMP1* expression by TNF-α (Panel B) and LPS (Panel C) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of four independent experiments **p ≤ 0.01.
Figure 3.11. Densitometric quantification of *MMP13* expression in C-20/A4 cells treated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 µg/ml) for 6 h.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) or LPS (0.1 – 1.0 µg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *MMP13* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with *β-actin* used as internal control (Panel A). Comparison of densitometrically quantified *MMP13* expression by TNF-α (Panel B) and LPS (Panel C) shown in arbitrary units, each value normalized to the respective *β-actin* expression. Data are presented as Mean ± SEM of four independent experiments **p ≤ 0.01, ***p ≤ 0.001.
Figure 3.12. Densitometric quantification of $MMP3$ expression in C-20/A4 cells treated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 µg/ml) for 6 h.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) or LPS (0.1 – 1.0 µg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for MMP3 was used to detect and quantify gene expression on 2 % agarose gels in triplicates with $\beta$-actin used as internal control (Panel A). Comparison of densitometrically quantified $MMP3$ expression by TNF-α (Panel B) and LPS (Panel C) shown in arbitrary units, each value normalized to the respective $\beta$-actin expression. Data are presented as Mean ± SEM of four independent experiments, **$p \leq 0.01$, ***$p \leq 0.001$. 

Panel A: Table showing the densitometric quantification of MMP3 expression in C-20/A4 cells treated with TNF-α (20.0 – 80.0 pg/ml) and LPS (0.1 – 1.0 µg/ml) for 6 h. The table includes the respective primers for MMP3 and $\beta$-actin, along with the corresponding bands on the gel and their respective bp size.

Panel B: Graph showing the normalized expression of MMP3 in response to different concentrations of TNF-α. The y-axis represents the normalized MMP3 expression, and the x-axis represents the concentration of TNF-α (in pg/ml).

Panel C: Graph showing the normalized expression of MMP3 in response to different concentrations of LPS. The y-axis represents the normalized MMP3 expression, and the x-axis represents the concentration of LPS (in µg/ml).
Figure 3.13. Densitometric quantification of MMP1, MMP3 and MMP13 expression in C-20/A4 cells treated with TNF-α (60.0 pg/ml) for 0-48 h.

C-20/A4 chondrocytes were stimulated with TNF-α (20.0 – 80.0 pg/ml) at time 0 and total RNA extracted 0-48 h post stimulation. PCR amplification with the respective primers for MMP1, MMP3 and MMP13 were used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified MMP1 (Panel B), MMP3 (Panel C) and MMP13 (Panel D) expression by TNF-α shown in arbitrary units, each value normalized to the respective β-actin expression. Dotted line represents basal expression levels. Data are presented as Mean ± SEM of four independent experiments *p≤ 0.05, ***p≤ 0.001.
3.1.4 Effect of TNF-α on COL2A1 and COL1A1 expression.

In healthy articular cartilage, chondrocytes are actively maintaining the steady-state expression of collagens and proteoglycans. Articular chondrocytes are sensitive to various growth factors and cytokines, which either enhance or reduce the synthesis of type II collagen, a marker of normal function of articular chondrocytes (Goldring et al., 1994). In osteoarthritis, cytokines including IL-1β, IL-6, IL-8, and TNF-α, produced by osteoarthritic chondrocytes significantly up-regulate matrix metalloproteinases (MMPs) gene expression, and decrease the synthesis of tissue specific macromolecules, such as collagen II, therefore inhibiting the chondrocyte’s compensatory synthesis pathways required to restore the integrity of the degraded extracellular matrix (Pelletier et al., 1991, Goldring et al., 1994b, Shlopov et al., 1997, Shlopov et al., 2000, Goldring et al., 2011).

Human C-20/A4 chondrocytes were found to respond to TNF-α in a time and concentration-dependent manner, by producing large amounts of the above-mentioned pro-inflammatory cytokines and significantly up-regulating collagenases 1 and 3. Next, in order to further investigate whether stimulation with TNF-α induces conditions resembling the events observed in OA chondrocytes, we investigated its effect on COL1A1, COL2A1 expression and additionally calculated the COL2A1:COL1A1 ratio to study the effect of TNF-α on the chondrocytic phenotype of the C-20/A4 cells. The results showed that expression of COL2A1, a marker for normal function of articular chondrocytes, was significantly down-regulated after TNF-α (60.0 pg/ml) treatment, in comparison to unstimulated cultures of C-20/A4 chondrocytes. The inflammogen caused a 66.7 % reduction in mRNA expression of collagen type II from 0.754 ± 0.08 au to 0.24 ± 0.012 au (p≤ 0.01) and 49 % reduction in COL1A1 expression from 2.25 ± 0.16 au to 1.121 ± 0.05 au (p≤ 0.01), as calculated following densitometric analysis (Figure 3.14 B) of the PCR product bands with the correct size (Figure 3.14 A).

Despite the large drop in overall collagen production, there was only slight, insignificant change in the indicator of differentiation COL2A1:COL1A1 ratio, as compared to untreated C-20/A4 chondrocytes, indicated in Figure 3.14 C.
Figure 3.14 Quantification of the effect of TNF-α on COL1A1 and COL2A1 expression.

C-20/A4 chondrocytes were treated with TNF-α (60.0 pg/ml) and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for COL1A1 and COL2A1 was used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified COL1A1 and COL2A1 expression (Panel B) is shown in arbitrary units (au), each value normalized to the respective β-actin expression. Comparison of COL2A1:COL1A1 ratio prior and after stimulation with TNF-α is shown in Panel C. Data are presented as Mean ± SEM of 3 independent experiments **p ≤ 0.01.
3.2 Effect of classical anti-inflammatory drugs on inflammatory mediator release from C-20/A4 chondrocytes

To determine whether inflammatory pathways were modulated in the presence of classical anti-inflammatory drugs, the effect of the non-steroidal anti-inflammatory drug indomethacin and glucocorticoid dexamethasone on stimulated chondrocytes was evaluated. Indomethacin is an indol derivative that's a non-selective COX-1 and COX-2 inhibitor with anti-inflammatory, analgesic, and antipyretic effects (Hart and Boardman, 1963). Dexamethasone is a potent synthetic member of the glucocorticoid class of steroid drugs. It acts as an anti-inflammatory and immunosuppressant drug and is 20-30 times more potent than the naturally occurring cortisol and 4-5 times more potent than prednisone (Vayssiere et al., 1997). Dexamethasone and indomethacin are used to treat many inflammatory and autoimmune diseases including osteoarthritis and rheumatoid arthritis.

3.2.1 Effect of indomethacin on IL-6 and IL-8 mRNA and protein in C-20/A4 chondrocytes.

C-20/A4 cell cultures were treated with 1.0 µM indomethacin for 30 min prior to 6 h incubation with TNF-α (60.0 pg/ml). Total RNA was extracted and PCR analysis performed using specific primers for the amplification of IL-6 and IL-8 (Table. 2.9). A significant decrease was observed in IL-6 and IL-8 expression following treatment with indomethacin (1.0 µM; Figure 3.15 A) with a significant 61.4 %, (p≤ 0.001) and 78.6 % (p≤ 0.001) down-regulation of IL-6 and IL-8 respectively as determined by densitometric quantification compared to β-actin (Figure 3.15 B).

Subsequently, its effect on IL-6 and IL-8 protein levels was determined. C-20/A4 chondrocytes were pre-treated with 1.0 µM indomethacin for 30 min prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected and analysed for IL-6 and IL-8 by ELISA. Indomethacin completely abrogated the release of the pro-inflammatory cytokines IL-6 and IL-8 bringing it down to basal levels (Figure 3.16 A and B).
**Figure 3.15. Densitometric quantification of the effect of indomethacin on TNF-α-mediated IL-6 and IL-8 gene expression.**

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or indomethacin (1.0 µM) prior to stimulation with TNF-α (60.0 pg/ml) and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for IL6 and IL8 was used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified IL6 and IL8 expression by TNF-α (Panel B) shown in arbitrary units, each value normalized to the respective β-actin expression. Data are presented as Mean± SEM of four independent experiments ***p≤ 0.001.
Figure 3.16. Effect of Indomethacin on TNF-α-mediated IL-6 and IL-8 release.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or Indomethacin (1.0 µM) prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected at 6 h post stimulation. IL-6 (Panel A) and IL-8 (Panel B) expression by TNF-α were then determined. Data are presented as Mean± SEM of n=4 independent experiments repeated in triplicate, **p≤ 0.01, ***p≤ 0.001.
3.2.2 Effect of dexamethasone on IL-6 and IL-8 mRNA and protein on C20/A4 chondrocyte.

In order to investigate the effect of dexamethasone on the expression of pro-inflammatory cytokines IL-6 and IL-8 in TNF-α treated C-20/A4 chondrocytes, cells were pre-treated with 1.0 µM dexamethasone for 30 mins before stimulation with TNF-α (60.0 pg/ml) and incubated for 6 h. PCR analysis was performed using specific primers for the amplification of IL-6 and IL-8 (Table. 2.9). Pre-treatment of C-20/A4 cells with 1.0 µM dexamethasone led to a significant 72.6 % down-regulation of IL-6 and IL-8 by 83 % compared to control (Figure 3.17). This effect at the mRNA level was confirmed at the protein level with a complete abrogation of TNF-α induced IL-6 and IL-8 release (Figure 3.18).
Figure 3.17. Densitometric quantification of the effect of dexamethasone on TNF-α-mediated IL6 and IL8 gene expression.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or dexamethasone (1.0 µM) prior to stimulation with TNF-α (60.0 pg/ml) and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for IL6 and IL8 was used to detect and quantify gene expression on 2% agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified IL6 and IL8 expression by TNF-α (Panel B) shown in arbitrary units, each value normalized to the respective β-actin expression. Data is presented as Mean ± SEM of n=4 independent experiments ***p≤ 0.001.
Figure 3.18. Effect of Dexamethasone on TNF-α-mediated IL-6 and IL-8 protein release.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or dexamethasone (1.0 µM) prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected at 6 h post stimulation. TNF-α stimulated IL-6 (Panel A) and IL-8 (Panel B) production was then determined. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
3.2.3 Effect of indomethacin on MMP1, MMP3 and MMP13 gene expression.

The effect of indomethacin on the regulation of MMP1, MMP3 and MMP13 gene expression in non-stimulated and TNF-α stimulated C-20/A4 chondrocytes was investigated (Figure 3.19).

C-20/A4 cells were pre-treated with 1.0 μM Indomethacin for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Total RNA was extracted and PCR analysis performed using specific primers for the amplification of MMP1, MMP3 and MMP13 (Table 2.9; Chapter 2). Following gel electrophoresis of the PCR reactions on 2 % agarose gels, the correct bands (Figure 3.19 A) were quantified densitometrically and a significant decrease in the proteinases expression (p ≤ 0.01) was detected with a >75 % inhibition of the interstitial collagenase MMP1, 67% reduction in MMP13 and a 49 % down-regulation of MMP3 expression compared to TNF-α stimulated controls (Figure 3.19 B).

3.2.4 Effect of dexamethasone on MMP1, MMP3 and MMP13 gene expression.

Following identification of the anti-cytokine properties of dexamethasone, its effect on the expression of MMP1, MMP3 and MMP13 in non-stimulated and TNF-α-treated C-20/A4 chondrocytes was investigated (Figure 3.20).

C-20/A4 cells were pre-treated with 1.0 μM dexamethasone for 30 minutes prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Total RNA was extracted and PCR analysis performed using specific primers for the amplification of MMP1, MMP3 and MMP13 (Table 2.9). A significant decrease in the proteinases expression (p ≤ 0.01) was detected with a >75 % inhibition of MMP1, 42 % reduction in MMP13 and a 49 % down-regulation of MMP3 expression compared to TNF-α stimulated controls (Figure 3.20).
Figure 3.19. Effect of Indomethacin on TNF-α-mediated MMP1, MMP3 and MMP13 expression.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or indomethacin (1.0 μM) prior to stimulation with TNF-α (60.0 pg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for MMP1, MMP3 and MMP13 were used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Densitometrically quantified MMP1, MMP3 and MMP13 expression by TNF-α alone or in presence of Indomethacin shown in arbitrary units, each value normalized to the respective β-actin expression (Panel B). Data are presented as Mean ± SEM of n=4 independent experiments *p≤ 0.05.
Figure 3.20. Effect of Dexamethasone on TNF-α-mediated MMP1, MMP3 and MMP13 expression.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS or dexamethasone (1.0 µM) prior to stimulation with TNF-α (60.0 pg/ml) at time 0 and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for MMP1, MMP3 and MMP13 were used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Densitometrically quantified MMP1, MMP3 and MMP13 expression by TNF-α alone or in presence of dexamethasone shown in arbitrary units, each value normalized to the respective β-actin expression (Panel B). Data are presented as Mean ± SEM of n=4 independent experiments *p≤ 0.05.
3.3 Effect of melanocortin peptides treatment on C-20/A4 activation.

3.3.1 Expression of MC₁, MC₃ and MC₅ mRNA and protein on C-20/A4 chondrocytic cells.

Melanocortin peptides (e.g. α-MSH) have been shown to possess anti-pyretic and anti-inflammatory functions via activation of a family of seven transmembrane receptors termed melanocortin receptors of which five have been identified (MC₁-₅, Getting et al., 2009). Expression of MC₁, MC₃ and MC₅ by C20/A4 chondrocytes was determined.

Total RNA was extracted from the cells and reverse-transcribed into cDNA via RT-PCR. PCR amplification reactions were used to assay for MC₁, MC₃ and MC₅ expression. MC₁ and MC₃ were highly expressed on C-20/A4 chondrocytes, with detection of specific bands corresponding to the expected size 493 bp and 820 bp respectively. Very slight MC₅ expression was also detected at the expected size of 340 bp. All samples were run alongside β-actin as an internal control (Figure 3.21).

Following identification of MC₁ and MC₃ mRNA, protein levels of these receptors were determined by employing Western Blotting. Protein extracts were prepared and examined for MC₁ and MC₃ protein expression. Western Blotting confirmed the presence of both of the melanocortin receptors in human C-20/A4 chondrocytes at the expected size of 35 and 40 kDa for MC₁ and MC₃ respectively. C-20/A4 cells expressed lower levels of MC₃ compared to MC₁ receptor after both were compared and normalized to the internal control α-Tubulin (Figure 3.22).
Figure 3.21. Endogenous expression of $MC_1$, $MC_3$ and $MC_5$ by C-20/A4 chondrocytic cell-line.

C-20/A4 chondrocytes were grown to confluence and RNA extracted. PCR amplification with the respective primers for $MC_1$, $MC_3$ and $MC_5$ were used to detect and quantify gene expression on 2% agarose gels in triplicates with $\beta$-actin used as internal control. Densitometrically quantified $MC_1$, $MC_3$ and $MC_5$ shown in arbitrary units, each value normalized to the respective $\beta$-actin expression. Data are presented as Mean ± SEM of 5 independent experiments.
Figure 3.22. Detection of MC$_1$ and MC$_3$ protein in human C-20/A4 chondrocytic cells.

Protein detection was performed using mouse anti-α-Tubulin mAb, rabbit anti-MC$_1$ mAb and rabbit anti-MC$_3$ mAb (1:2000). Bands with sizes corresponding to MC$_1$ (35 kDa), MC$_3$ (40 kDa) and α-tubulin (55 kDa) were detected and melanocortins receptor expression confirmed in C-20/A4 cells. The image is a representative of 3 individual experiments.
3.3.2 cAMP accumulation in C-20/A4 chondrocytes following melanocortin peptide stimulation.

Following identification of $MC_1$, $MC_3$ and $MC_5$ mRNA and $MC_1$, $MC_3$ protein, a panel of melanocortin peptides was evaluated to ascertain receptor functionality by determining intracellular cAMP accumulation within the C20/A4 chondrocytes. C-20/A4 cells were treated with $\alpha$-MSH, D[TRP]$^8$-$\gamma$-MSH, SHU9119, PG910, PG911 or a direct adenylate cyclase stimulator, forskolin, used as positive control in all cases. Forskolin (3.0 $\mu$M) increased the intracellular cAMP level to 2227.44 ± 74.42 fmol/well, representing 9-fold increase over PBS-vehicle control (249.36 ± 10.56 fmol/well). Treatment of C-20/A4 chondrocytes with the pan melanocortin receptor agonist $\alpha$-MSH (1.0 – 30.0 $\mu$g/ml), for 30 mins caused a significant increase in intracellular cAMP at 3.0, 10.0 and 30.0 $\mu$g/ml by approximately 2-fold, 2.6-fold and 1.7-fold increase to 568.86 ± 22.74 fmol/well ($p \leq 0.001$), 638.6 ± 41.6 fmol/well ($p \leq 0.001$) and 429.1 ± 18.9 fmol/well ($p \leq 0.05$) respectively compared to the vehicle control level of 249.36 ± 10.56 fmol/well (Figure 3.23 A).

Similarly, a drastic 3.2-fold increase in cAMP accumulation peaking at 800.83 ± 30.0 fmol/well ($p \leq 0.001$) was recorded following treatment with 3.0 $\mu$g/ml of the highly selective $MC_3$ receptor agonist D[TRP]$^8$-$\gamma$-MSH (Figure 3.23 B). A significant increase of 3-fold compared to vehicle control levels was observed at 10.0 $\mu$g/ml (789.9 ± 49.15 fmol/well, $p \leq 0.001$) and 30.0 $\mu$g/ml (696.2 ± 27.59 fmol/well, $p \leq 0.001$). In comparison, the $MC_{3/4}$ receptor antagonist SHU9119 (1.0 – 10.0 $\mu$g/ml) failed to induce a statistically significant cAMP response in C-20/A4 cells at any of the concentrations tested (Figure 3.23 C).

The $MC_5$ full agonists PG901 and PG911 (1.0 –10.0 $\mu$g/ml) were then evaluated to determine whether $MC_5$ was a functionally active receptor. Both are full agonists at the $MC_5$ (EC(50) = 0.072 nM and 0.031 nM, respectively), but full antagonists at the $MC_{3/4}$. However, no statistically significant cAMP formation was recorded following stimulation at any of the concentrations (Figure 3.23 D, E).
Following identification that \( \alpha \)-MSH and \( \text{D}[\text{TRP}]^8\text{-\( \gamma \)}\)-MSH induced cAMP accumulation in C-20/A4 chondrocytes, the peptides were evaluated in the presence of the MC\(_{3/4} \) antagonist SHU9119 to determine if the cAMP accumulation occurred via these receptors (Figure 3.24). C-20/A4 chondrocytes were incubated with \( \alpha \)-MSH and \( \text{D}[\text{TRP}]^8\text{-\( \gamma \)}\)-MSH (0 – 30.0 \( \mu \)g/ml) alone or in the presence of SHU9119 (10.0 \( \mu \)g/ml).

SHU9119 totally abrogated the effect elicited by \( \text{D}[\text{TRP}]^8\text{-\( \gamma \)}\)-MSH, whilst cAMP formation triggered by \( \alpha \)-MSH was only slightly reduced at 3.0 \( \mu \)g/ml (Figure 3.24 B). SHU9119 inhibited the activation of the MC\(_3 \) elicited by \( \text{D}[\text{TRP}]^8\text{-\( \gamma \)}\)-MSH at all concentrations evaluated; where at 3.0 \( \mu \)g/ml of the antagonist, only slight inhibition resulted leading it to drop from 379.82 ± 15.29 fmol/well to 320.21 ± 30.35 fmol/well (n/s, \( p > 0.05 \)). The addition of the antagonist with 3.0 \( \mu \)g/ml \( \text{D}[\text{TRP}]^8\text{-\( \gamma \)}\)-MSH caused the cAMP levels to fall from 800.83 ± 29.99 to 318.84 ± 9.34 fmol/well, representing ~ 87 % reduction (\( p \leq 0.001 \)).

Similarly, SHU9119 (10.0 \( \mu \)g/ml) inhibition led to a >84 % reduction in cAMP accumulation elicited by 10.0 \( \mu \)g/ml \( \text{D}[\text{TRP}]^8\text{-\( \gamma \)}\)-MSH (\( p \leq 0.01 \)) with a reduction from 789.9 ± 49.2 fmol/well down to 338.04 ± 10.6 fmol/well cAMP accumulation for \( \text{D}[\text{TRP}]^8\text{-\( \gamma \)}\)-MSH and \( \text{D}[\text{TRP}]^8\text{-\( \gamma \)}\)-MSH + SHU9119 (10.0 \( \mu \)g/ml). Even the highest concentration of the selective MC\(_3 \) agonist, had no effect when given simultaneously with the MC\(_{3/4} \) receptor antagonist: the concentration of cAMP was reduced from 696.21 ± 27.59 to 290.12, illustrating an 80 % reduction in cAMP accumulation (\( p \leq 0.001 \)). cAMP levels triggered by \( \alpha \)-MSH, were not reduced by SHU9119, except when the antagonist was added in conjunction with 3.0 \( \mu \)g/ml of \( \alpha \)-MSH, when SHU9119 (10.0 \( \mu \)g/ml) led to 15.5% decrease in cAMP formation from 568.86 ± 22.74 to 480.29 ± 35.3 (n.s., \( p > 0.05 \); Figure 3.24 A).
Figure 3.23. MC₁, MC₃, MC₅ functionality determined by cAMP EIA.
C-20/A4 chondrocytes were treated with 1.0-30.0 µg/ml α-MSH (A), [DTRP⁸]-γ-MSH (B) and with 1.0-10.0 µg/ml SHU9119 (C), PG-901 (D) and PG911 (E) for 30 min and cAMP (fmoles/well) accumulation determined by cAMP EIA. Dotted lines indicate basal accumulation in PBS-treated whilst dashed lines indicate maximal accumulation in 3.0 µM FSK-treated C20/A4 cells. Data is Mean ± SEM of n=6 samples, n.s. p > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001) vs. PBS-treated control cultures.
Figure 3.24. SHU9119 prevents D[TRP]$^8$-$\gamma$-MSH from activating MC$_3$, but has no effect on MC$_1$ functionality.

C-20/A4 chondrocytes were treated with 1.0-30.0 µg/ml $\alpha$-MSH (Panel A) and D[TRP]$^8$-$\gamma$-MSH (Panel B) alone or in the presence of SHU9119 (10 µg/ml) for 30 min and cAMP (fmoles/well) accumulation determined by cAMP EIA. Dotted lines indicate basal accumulation in PBS-treated, whilst dashed lines indicate maximal accumulation in FSK-treated C20/A4 cells. Data is Mean ± SEM of n=6 samples, $^* p \leq 0.05$, $^{**} p \leq 0.01$ vs. PBS-treated control cultures.
3.3.3 Modulation of pro-inflammatory mediator release and apoptosis by melanocortin peptides on TNF-α stimulated C20/A4 chondrocytes.

3.3.3.1 Effect of melanocortin peptides on basal release of pro-inflammatory cytokines production from C-20/A4 chondrocytes.

Following identification of functionally active MC₁ and MC₃ on C-20/A4 chondrocytes, their effect on basal cytokine production was determined. C-20/A4 chondrocytes were treated with α-MSH (3.0 µg/ml), D[TRP]₈-γ-MSH (3.0 µg/ml) and SHU9119 (10.0 µg/ml), and the basal levels of production of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and MCP-1 were determined by cytokine ELISAs (Table 3.3). Treatment of cells with melanocortin peptides did not cause significant alteration compared to control cultures treated with DMEM alone (p>0.05).

Table 3.3 Effect of melanocortin derived peptides on basal levels of pro-inflammatory cytokine release from C-20/A4 chondrocytes.

<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM Media</td>
<td>2.77±0.44</td>
<td>21.09±18.5</td>
<td>72.65±4.78</td>
<td>46.18±14.12</td>
</tr>
<tr>
<td>α-MSH (3.0 µg/ml)</td>
<td>5.76±1.8</td>
<td>38.29±29.1</td>
<td>82.3±5.36</td>
<td>54.26±11.11</td>
</tr>
<tr>
<td>D[TRP]₈-γ-MSH (3.0 µg/ml)</td>
<td>3.69±0.65</td>
<td>23.19±11.65</td>
<td>67.76±6.3</td>
<td>48.42±7.1</td>
</tr>
<tr>
<td>SHU9119 (10.0 µg/ml)</td>
<td>4.55±0.55</td>
<td>37.19±2.97</td>
<td>85.58±4.85</td>
<td>54.41±11.93</td>
</tr>
</tbody>
</table>

Data are Means ± SEM of n=4 of three determinations. Interleukin(IL)-1β; IL-6, Interleukin(IL)-6, interleukin(IL)-8; MCP-1, monocyte chemoattractant protein-1
3.3.3.2 Attenuation of TNF-\(\alpha\)-activated pro-inflammatory cytokines release from C-20/A4 chondrocytes by \(\alpha\)-MSH and D[TRP]\^8-\(\gamma\)-MSH.

The identification of the melanocortin receptors’ functionality on C-20/A4 chondrocytes and their non-cytotoxic effects, led to the evaluation of \(\alpha\)-MSH and D[TRP]\^8-\(\gamma\)-MSH effect on pro-inflammatory cytokine release following TNF-\(\alpha\)-stimulation.

The C-20/A4 cell-line was treated with \(\alpha\)-MSH or D[TRP]\^8-\(\gamma\)-MSH in concentrations ranging from 0.3 – 30.0 \(\mu\)g/ml for 30 mins prior to stimulation with TNF-\(\alpha\), cell free supernatants were collected at 2, 6 and 24 h post-stimulation and pro-inflammatory cytokines IL-1\(\beta\), IL-6 and IL-8 were determined by commercially available ELISAs.

\(\alpha\)-MSH led to a significant concentration-dependent decrease in IL-1\(\beta\) concentration with a maximal reduction detected at 3.0 \(\mu\)g/ml \(\alpha\)-MSH with 88.6 ± 3.2 % at 2 h (\(p\leq 0.001\)) and 84.8 ± 4.01 % at 6 h (\(p\leq 0.001\)) compared to TNF-\(\alpha\)-stimulated controls. The melanocortin peptide did not modulate IL-1\(\beta\) release at 24 h post-stimulation (Figure 3.25 A). The MC\(_3\) agonist D[TRP]\^8-\(\gamma\)-MSH (3.0 \(\mu\)g/ml) led to the maximal observed reduction of 72.73 ± 3.31 % at 6 h (Figure 3.25 B).

Evaluating the effects of \(\alpha\)-MSH on IL-6 showed that after 6 h, \(\alpha\)-MSH caused a concentration-dependant effect peaking at 3.0 \(\mu\)g/ml with 72.1 ± 2.3 % compared to TNF-\(\alpha\)-stimulated controls. It was observed that higher concentrations did not elicit a significant effect. \(\alpha\)-MSH did not exert any detectable effect at 24 h post-stimulation, except when C-20/A4 chondrocytes were treated with low concentration of 0.3 \(\mu\)g/ml with 37 ± 3.4 % and 34.5 ± 4.6 % inhibition of IL-6 respectively (\(p\leq 0.05\)). D[TRP]\^8-\(\gamma\)-MSH caused a concentration-dependent response observed at all time-points (Figure 3.26 B). The maximum effect at 2 h was observed following stimulation with 0.3 and 3.0 \(\mu\)g/ml with 75.0 ± 6.9 % and 73.1 ± 3.5 % reduction respectively. At 6 h, 3.0 \(\mu\)g/ml were identified as the most potent concentrations leading to 61.3 ± 2.1 % and 60.2 ± 2.6 % inhibition of IL-6.
synthesis respectively. The peptide was still effective at 24 h post stimulation with a pronounced concentration-dependent reduction of IL-6 concentration, peaking with 85 ± 2.0 % inhibition at 30.0 µg/ml (Figure 3.26 B).

α-MSH inhibited IL-8 release in a concentration dependent manner at 2 and 6 h and was not effective at later time points (Figure 3.27 A). At 2 h, the greatest reduction in IL-8 synthesis was caused by 3.0, and 30.0 µg/ml of α-MSH with 59.2 ± 5.5 % and 66.7 ± 9.85 % decrease respectively, while 0.3 had less effect leading to 47.2 ± 9.6 % down-regulation. There was a concentration dependent inhibition of IL-8, 6 h post stimulation, with a maximal reduction of 60.21 ± 2.1% caused by 3.0 µg/ml α-MSH.

D[TRP]⁸-γ-MSH (0.3 µg/ml) failed to suppress the effect of TNF-α, except at 24 h post-stimulation, when it caused 51% decrease in the cytokine production (Figure 3.27 B). The higher concentration of 3.0 µg/ml, led to significant down-regulation of IL-8 release regardless of the time point evaluated with a 67.32 ± 2.76%, 44.71 ± 5.14 % and 37.11 ± 2.4 % reductions at 2, 6 and 24h respectively. Following treatment of C-20/A4 chondrocyte with 30.0 µg/ml of D[TRP]⁸-γ-MSH a consistent decrease of 80.72 ± 2.13 % (p≤ 0.01), 64.72 ± 2.3 % (p≤ 0.001) and 69.89 ± 3.23 % (p≤ 0.01) was observed at 2, 6 and 24 h post-stimulation respectively.
Figure 3.25. Effect of α-MSH and D[TRP]^8-γ-MSH on TNF-α induced IL-1β release from C-20/A4 cells.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS (Dotted line), α-MSH or D[TRP]^8-γ-MSH (0.3 – 30.0 µg/ml) prior to stimulation with TNF-α (60.0 pg/ml; dashed line) and cell-free supernatants collected at 2, 6 and 24 h post stimulation and analysed for IL-1β by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Figure 3.26. Effect of α-MSH and D[TRP]8-γ-MSH on TNF-α induced IL-6 release from C-20/A4 cell-line.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS (Dotted line), α-MSH or D[TRP]8-γ-MSH (0.3 – 30.0 µg/ml) prior to stimulation with TNF-α (60.0 pg/ml; dashed line) and cell-free supernatants collected at 2, 6 and 24 h post stimulation and analysed for IL-6 by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, **p≤ 0.01, ***p≤0.001.
Figure 3.27. Effect of α-MSH and D[TRP]^8-γ-MSH on TNF-α induced IL-8 release from C-20/A4 cell-line.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS, α-MSH (Panel A) and D[TRP]^8-γ-MSH (0.3 – 30.0 µg/ml, Panel B) prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected at 0, 2, 6 and 24 h post stimulation and analysed for IL-8 by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
### 3.3.3.3 Effect of PG901 and PG911 on TNF-α stimulated pro-inflammatory cytokines release from C-20/A4 chondrocytes.

Following the finding that MC₅ was not functionally active, the effects of the selective agonist PG901 and PG911 on cytokine release was evaluated to ensure that the anti-cytokine effects elicited by α-MSH were independent of MC₅. C-20/A4 chondrocytes were pre-treated with PG901 or PG911 (3.0 or 10.0 µg/ml) prior to stimulation with TNF-α (60.0 pg/ml) and were incubated for 6 h. Subsequently, IL-1β, IL-6, IL-8 and MCP-1 release was determined by ELISA (Table 3.1). As previously observed (Figures 3.5, 3.6 and 3.8), TNF-α (60.0 pg/ml) caused a significant release of these cytokines compared to control cells (†p≤ 0.05). IL-1β, IL-6 and MCP-1 were not inhibited by PG901 or PG911 at any concentration evaluated. However, a different scenario was observed with respect to IL-8 only PG911 (10.0 µg/ml) caused a 40.4 ± 23.3 % reduction; all other concentrations of PG911 and PG901 were inactive.

### Table 3.1 Effect of PG901 and PG911 on TNF-α induced cytokine release from C-20/A4 cell-line.

<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>2.77± 0.44</td>
<td>21.09±18.5</td>
<td>72.65±4.78</td>
<td>46.18 ± 14.12</td>
</tr>
<tr>
<td>TNF-α (60 pg/ml)</td>
<td>24.37±8.69†</td>
<td>280.6 ± 20.39†</td>
<td>202.82 ± 21.04†</td>
<td>101.2± 16.37†</td>
</tr>
<tr>
<td>TNF-α + PG901 (3.0 µg/ml)</td>
<td>17.56 ± 1.03</td>
<td>231.29 ± 24.19</td>
<td>165.41 ± 23.81</td>
<td>84.6 ± 16.37</td>
</tr>
<tr>
<td>TNF-α + PG901 (10.0 µg/ml)</td>
<td>19.43 ± 3.29</td>
<td>253.62 ± 26.46</td>
<td>144.84 ± 25.41</td>
<td>89.96 ± 18.91</td>
</tr>
<tr>
<td>TNF-α + PG911 (3.0 µg/ml)</td>
<td>19.92 ±4.99</td>
<td>273.396 ± 48.21</td>
<td>181.37 ± 54.78</td>
<td>97.05 ± 5.29</td>
</tr>
<tr>
<td>TNF-α + PG911 (10.0 µg/ml)</td>
<td>16.38 ± 2.01</td>
<td>244.29 ± 17.14</td>
<td><strong>122.46 ± 18.47</strong>*</td>
<td>85.88 ± 6.05</td>
</tr>
</tbody>
</table>

Data are Means ± SEM of n=4 of three determinations †, *p≤ 0.05.

IL-1β, Interleukin 1β; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1.
3.3.4 Effect of SHU9119 on α-MSH and D[TRP]8-γ-MSH modulation of pro-inflammatory cytokines from C-20/A4 chondrocytes.

The effect of the MC3/4 antagonist SHU9119 on α-MSH and D[TRP]8-γ-MSH modulation of cytokine release was evaluated to determine whether MC1 or MC3 were transmitting the anti-inflammatory effects of these peptides in the chondrocyte system. C-20/A4 chondrocytes were pre-treated with α-MSH or D[TRP]8-γ-MSH (3.0 µg/ml) ± SHU9119 (10.0 µg/ml) for 30 mins prior to stimulation with TNF-α (60.0 pg/ml) and total RNA and cell-free supernatants were collected at 6 h post-stimulation. Total RNA was extracted from the treated C-20/A4 chondrocytes and PCR amplification performed using IL6 and IL8 specific primers (Table 2.9).

3.3.4.1 Antagonistic effect of SHU9119 on IL6 and IL8 gene expression following α-MSH and D[TRP]8-γ-MSH stimulation

Densitometric quantification (Figure 3.28 A) showed that α-MSH (3.0 µg/ml) significantly down-regulated TNF-α-induced IL6 and IL8 expression, therefore causing a 35 % drop in IL6 expression from 0.802 ± 0.025 to 0.517 ± 0.018 au ($p ≤ 0.05$) and a 24.5 % down-regulation of IL-8 from 1.92 ± 0.1 au down to 1.45 ± 0.028 au ($p ≤ 0.05$), this effect was not blocked by SHU9119 (Figure 3.28 B and C).

The MC3 agonist D[TRP]8-γ-MSH significantly inhibited the transcription of both IL6 and IL8 genes as shown by densitometric quantification (Figure 3.29 A) with a 27 % inhibition of IL6 expression from 0.802 ± 0.025 au to 0.585 ± 0.02 au ($p ≤ 0.05$) and 35.7 % reduction in IL8 transcript from 1.92 ± 0.1 to 1.234 ± 0.016 au ($p ≤ 0.01$). SHU9119 completely inhibited the effect of D[TRP]8-γ-MSH on IL6 and IL8, such that IL6 expression was 0.94 ± 0.05 au and IL-8 was 1.85 ± 0.19 au ($p ≤ 0.05$; Figure 3.29 B and C).
3.3.4.2 Effect of SHU9119 on cytokine release from $\alpha$-MSH and \(D[\text{TRP}]^8\)-\(\gamma\)-MSH pre-treated TNF-$\alpha$-activated C-20/A4 chondrocytes.

In order to further elucidate the function of the melanocortin peptides $\alpha$-MSH and \(D[\text{TRP}]^8\)-\(\gamma\)-MSH on TNF-$\alpha$ stimulated cytokine production, the MC$_{3/4}$ antagonist SHU9119 was used to selectively block the function of MC$_3$. C-20A4 cells were pre-treated with 3.0 $\mu$g/ml of $\alpha$-MSH or \(D[\text{TRP}]^8\)-\(\gamma\)-MSH for 30 min prior to stimulation with TNF-$\alpha$ (60.0 pg/ml) for 6 h and IL-6, IL-8 and MCP-1 levels were detected by ELISA. $\alpha$-MSH (3.0 $\mu$g/ml) caused a marked reduction in IL-6, causing a 72.13 ± 2.3 % ($p \leq 0.01$; Figure 3.30 A), 60.22% ($p \leq 0.01$) reduction in IL-8 production (Figure 3.30 B) and a 21.3 % reduction in MCP-1 ($p \leq 0.05$; Figure 3.30C). These effects were not abrogated in the presence of SHU9119 (Figure 3.30 A-C).

The selective MC$_3$ agonist \(D[\text{TRP}]^8\)-\(\gamma\)-MSH inhibited IL-6 production by 68 % ($p \leq 0.01$), 44.72 % ($p \leq 0.01$) reduction in IL-8 secretion and a 26 % ($p \leq 0.01$) reduction in MCP-1 ($p \leq 0.05$) an effect blocked by SHU9119 (Figure 3.30 D-F).
Figure 3.28. Densitometric quantification of *IL-6* and *IL-8* mRNA expression in TNF-α-activated C-20/A4 chondrocytes treated with α-MSH and SHU9119 for 6 h. C-20/A4 chondrocytes were pre-treated for 30 min with α-MSH (3.0 µg/ml) ± SHU9119 (10.0 µg/ml) prior to stimulation with TNF-α (60.0 pg/ml) and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *IL-6* and *IL-8* was used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified *IL-6* and *IL-8* expression following treatments (Panel B) shown in arbitrary units, each value normalized to the respective β-actin expression. Data are presented as Mean ± SEM of n=4 independent experiments *p≤ 0.05.
Figure 3.29. Densitometric quantification of IL-6 and IL-8 mRNA expression in TNF-α-activated C-20/A4 chondrocytes treated with D[TRP]$^8$-γ-MSH and SHU9119 for 6 h.

C-20/A4 chondrocytes were pre-treated for 30 min with D[TRP]$^8$-γ-MSH (3.0 µg/ml) ± SHU9119 (10.0 µg/ml) prior to stimulation with TNF-α (60.0 pg/ml) and total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for IL-6 and IL-8 was used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified IL-6 and IL-8 expression following treatments (Panel B) shown in arbitrary units, each value normalized to the respective β-actin expression. Data are presented as Mean ± SEM of n=4 independent experiments *$p \leq 0.05$, **$p \leq 0.01$.)
Figure 3.30. Effect of SHU9119 on IL-6, IL-8 and MCP-1 release from α-MSH and D[TRP]8-γ-MSH pre-treated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS, α-MSH (Panel A – C) or D[TRP]8-γ-MSH (3.0 µg/ml) alone (Panels D – F) or in the presence of SHU9119 (10.0 µg/ml) prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected at 6 h post stimulation and analysed for IL-6, IL-8 and MCP-1 by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p ≤ 0.05, **p ≤ 0.01.
3.3.4.3 Effect of SHU9119 on *MMP1*, *MMP3* and *MMP13* expression in α-MSH D[TRP]^{8}-γ-MSH and pre-treated TNF-α activated chondrocytes.

Following the identification of the anti-cytokines effects of α-MSH and D[TRP]^{8}-γ-MSH, their effect was evaluated on matrix metalloproteinases expression (Figure 3.31A). α-MSH (3.0 µg/ml) caused a significant ∼80 % down-regulation in the transcription of *MMP1*; densitometric quantification of the bands revealed that expression was reduced from 1.71 ± 0.11 au to 0.34 ± 0.04 au (p≤ 0.001; Figure 3.31 B). *MMP3* expression was reduced by α-MSH by 48 % from 0.37 ± 0.02 au (TNF-α-treated control cultures) to 0.19 ± 0.03 au (p≤ 0.01; Figure 3.31C), whilst *MMP13* expression was inhibited by 66.7 % from 0.24 ± 0.01 au (TNF-α, 60.0 pg/ml) to 0.083 ± 0.002 au (Figure 3.31 D).

This inhibition of *MMP1*, *MMP3* and *MMP13* was not modified in the presence of SHU9119. Nevertheless, when SHU9119 (10.0 µg/ml) was added together with α-MSH (3.0 µg/ml), there was a marked synergistic down-regulation of *MMP13* synthesis, with the combination causing a marked inhibition of 87.5 % (p≤ 0.001) compared to TNF-α treated controls and 57.8 % drop compared to samples pre-treated with α-MSH alone (p≤ 0.05).

Treatment of C-20/A4 chondrocytes with D[TRP]^{8}-γ-MSH (3.0 µg/ml) led to a significant 88.9 % reduction in *MMP1* expression (0.189 ±0.1 au; p≤ 0.001; Figure 3.32A) an effect blocked completely by co-administration of SHU9119 (10.0 µg/ml; Figure 3.32 B). *MMP3* was reduced by 76 % (0.098 ± 0.006 au; p≤ 0.01) compared to TNF-α stimulated cultures (0.372 ± 0.012 au) an effect blocked by SHU9119 (10.0 µg/ml), returning the expression levels back to TNF-α-stimulated levels (0.372 ± 0.012 au; Figure 3.32 C). A similar observation was noted for *MMP13* gene expression, where D[TRP]^{8}-γ-MSH (3.0 µg/ml) caused a 91.7 % drop in transcription levels (0.02 ± 0.004 au; p≤ 0.001) as compared to TNF-α–treated controls (0.237 ± 0.008 au), an effect completely abolished by SHU9119 (10.0 µg/ml; Figure 3.32 D).
Figure 3.31. Densitometric quantification of MMPs mRNA expression in C-20/A4 cells treated with α-MSH and SHU9119 for 6 h.

C-20/A4 chondrocytes were pre-treated for 30 min with α-MSH (3.0 µg/ml) ± SHU9119 (10.0 µg/ml) and stimulated with TNF-α (60.0 pg/ml). Total RNA was extracted at 6 h post stimulation and PCR amplification with the respective primers for MMP1, MMP3 and MMP13 was used to detect and quantify gene expression on 2 % agarose gels in triplicates, with β-actin used as internal control (Panel A). Comparison of densitometrically quantified MMP1, MMP3 and MMP13 expression for α-MSH ± SHU9119 (Panel B, C and D) shown in arbitrary units, each value normalized to the respective β-actin expression. Data is presented as Mean ± SEM of n=4 independent experiments *p≤0.05, **p≤0.01, ***p≤0.001).
Figure 3.32. Densitometric quantification of MMPs mRNA expression in C-20/A4 cells treated with D[TRP]$^8$-γ-MSH and SHU9119 for 6 h.

C-20/A4 chondrocytes were pre-treated for 30 min with D[TRP]$^8$-γ-MSH (3.0 µg/ml) ± SHU9119 (10.0 µg/ml) and stimulated with TNF-α (60.0 pg/ml). Total RNA was extracted at 6 h post stimulation and PCR amplification with the respective primers for MMP1, MMP3 and MMP13 was used to detect and quantify gene expression on 2 % agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified MMP1, MMP3 and MMP13 expression for [DTRP]$^8$-γ-MSH ± SHU9119 (Panel B, C and D) shown in arbitrary units, each value normalized to the respective β-actin expression. Data is presented as Mean ± SEM of n=4 independent experiments *p≤0.05, **p≤ 0.01, ***p≤ 0.001).
3.3.5 Evaluation of the effect of melanocortin peptides on anti-inflammatory protein synthesis and release from C-20/A4 chondrocytes.

Human chondrocytes from healthy and osteoarthritic cartilage have been previously shown to express both IL-10 as well as the IL-10 receptor (IL10R; Iannone et al., 2001). IL-10 has potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1β by activated cells. Direct stimulation of OA chondrocytes with IL-10 has been shown to inhibit the activation of chondrocytes by TNF-α and therefore to down-regulate the expression of MMP1 and MMP13 (Shlopov et al., 2000). In addition, IL-10 has been shown to induce HO-1, which is implicated in the protection against tissue damage and is repressing pro-inflammatory cytokines such as TNF-α. Therefore, following identification of the anti-inflammatory properties of α-MSH and D[TRP]8-γ-MSH on TNF-α stimulated C20/A4 chondrocytes, their effect on the production of these anti-inflammatory proteins was next evaluated.

3.3.5.1 Effect of melanocortin peptides alone on IL-10 release from C-20/A4 chondrocytes.

IL-10 protein levels in cell-free supernatants were determined by ELISA following stimulation of C-20/A4 chondrocytes with a panel of melanocortin peptides (α-MSH, D[TRP]8-γ-MSH, PG901, PG911 all at 3.0 µg/ml and SHU9119 (10.0 µg/ml) for 0, 2, 6 and 24 h.

Figure 3.33 A and B demonstrates the time-dependent increase in IL-10 release peaking at 6 h following α-MSH (3.0 µg/ml) and D[TRP]8-γ-MSH (3.0 µg/ml) treatment. At time 0 there was almost no basal release of IL-10 (1.625 ± 0.92 pg/ml). However, following stimulation with α-MSH and D[TRP]8-γ-MSH for 2 h, there was a significant 15.5-fold (25.28 ± 2.25 pg/ml; *p≤ 0.05) and 12-fold (19.96 ± 3.25 pg/ml; *p≤ 0.05) increase in IL-10 production for both peptides respectively. IL-10 levels steadily increased to 37.9 ± 4.13 pg/ml and 25.92 ± 2.3 pg/ml following stimulation with α-MSH and D[TRP]8-γ-MSH for 6 h respectively. At 24 h post-treatment, α-MSH and D[TRP]8-γ-MSH treatment caused IL-10 concentrations to return to levels similar to those observed at 2h (15.67 ± 3.93...
pg/ml and 19.23 ± 4.76 pg/ml, *p ≤ 0.05 both peptides). At all time points SHU9119, PG901 and PG911 (10.0 µg/ml) failed to induce any significant increase in IL-10 above basal production at time 0h (Figure 3.33 C-E).

3.3.5.2 Effect of melanocortin peptides on IL-10 production from TNF-α-activated C-20/A4 chondrocytes.

Pre-treatment of TNF-α-activated C-20/A4 chondrocytes with α-MSH (0.3 – 30.0 µg/ml) for 2h caused a concentration-dependent increase in IL-10, peaking with 37.5 ± 2.83 pg/ml following addition of 3.0 µg/ml α-MSH. Concentrations higher than 3.0 µg/ml didn’t cause significant increases in IL-10 compared to untreated controls (1.625 ± 0.919 pg/ml). At 6 h post stimulation α-MSH caused a significant increase in IL-10 production at 0.3 – 3.0 µg/ml (*p ≤ 0.05) compared to untreated controls (5.34 ± 3.34 pg/ml). The maximal response of 48.42 ± 3.21 pg/ml was elicited by 0.3 µg/ml α-MSH, although this was not significantly different compared to 3.0 µg/ml (43.83 ± 2.24 pg/ml, p ≤ 0.05; Figure 3.34). IL-10 release was subsequently evaluated at 24 h, in order to determine if this induction was maintained over a longer time frame. At this time-point a different scenario was observed with concentrations of 3.0 and 30.0 µg/ml able to cause detectable IL-10 release. The maximal response at this time-point was elicited by 30.0 µg/ml α-MSH (50.19 ± 4.12 pg/ml).

Given that the pan-melanocortin agonist α-MSH caused increases in IL-10 the MC₃ agonist D[TRP]₈-γ-MSH (0.3 - 30.0 µg/ml) was subsequently evaluated prior to stimulation with TNF-α (60.0 pg/ml). The MC₃ agonist caused a marked release of IL-10 at 2, 6 and 24 h post stimulation, with concentrations of 3.0 and 30.0 µg/ml proving to be the most effective. D[TRP]₈-γ-MSH (30.0 µg/ml) caused a maximal release of 34.12 ± 2.52 pg/ml of IL-10 at 2 h, compared to untreated controls (1.625 ± 0.919 pg/ml; *p ≤ 0.05). At 6 h post stimulation 3.0 and 30.0 µg/ml of D[TRP]₈-γ-MSH significantly increased IL-10 release with a peak of 24.94 ± 2.15 pg/ml at 3.0 µg/ml. At 24 h a concentration-dependent increase in IL-10 release was observed with a maximal release of 37.33 ± 0.83 pg/ml at 3.0 µg/ml (Figure 3.34).
Figure 3.33. Time dependent release of IL-10 from C20/A4 cells following treatment with α-MSH, D[TRP]β-γ-MSH, SHU9119, PG901 and PG911.

C-20/A4 chondrocytes were treated α-MSH (3.0 µg/ml, Panel A), D[TRP]β-γ-MSH (3.0 µg/ml; Panel B), SHU9119 (10.0 µg/ml; Panel C), PG901 (3.0 µg/ml; Panel D) or PG911 (3.0 µg/ml; Panel E) and cell-free supernatants collected at 0, 2, 6 and 24 h. IL-10 was then determined by ELISA. Data are presented as Mean± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, vs. Time 0 (dotted line).
Figure 3.34 Effect of α-MSH and D[TRP]₈-γ-MSH on IL-10 release from TNF-α-activated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were treated with α-MSH or D[TRP]₈-γ-MSH (0.3 – 30.0 µg/ml) for 30 min prior to TNF-α (60.0 pg/ml) stimulation. Cell-free supernatants were collected at 2, 6 and 24 h and analysed for IL-10 by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicates. *p≤ 0.05, **p≤ 0.01 vs. untreated control cultures.
3.3.5.3 Determination of HO-1 release from C-20/A4 cells following melanocortin peptide stimulation.

HO-1 release from C-20/A4 chondrocytes treated with vehicle (DMEM), α-MSH or D[TRP]γ-MSH (3.0 µg/ml) alone or in presence of TNF-α (60.0 pg/ml) was investigated via Western Blot and the results are shown in Figure 3.35. C-20/A4 chondrocytes were treated with melanocortin peptides for 6 h either alone or given 30 min prior to stimulation with TNF-α and samples collected 6 h later.

Western Blot analysis identified basal production of HO-1 (0.50 ± 0.04 au), which was significantly increased following treatment with TNF-α (60.0 pg/ml) for 6 h to 1.02 ± 0.01 au (p≤ 0.001). The treatment of chondrocytes with α-MSH (3.0 µg/ml) alone caused a significant 3-fold increase in HO-1 production (1.39 ± 0.04 au; p≤ 0.001), compared to the media-treated controls and a 36 % increase when compared to TNF-α alone (p≤ 0.01). The combination of α-MSH and TNF-α caused a significant increase in HO-1 with a 6-fold increase in HO-1 (2.64 ± 0.14 au, p≤ 0.001) compared to untreated controls and an approximate 3-fold increase when compared to TNF-α-stimulated samples (p≤ 0.001).

A similar observation was made following treatment of the cells with the MC₃ agonist D[TRP]γ-MSH (3.0 µg/ml), which caused a 3-fold increase in HO-1 expression, raising it to 1.37 ± 0.02 au (p≤ 0.01) compared to untreated controls and 34 % increase compared to HO-1 production by TNF-α-stimulated chondrocytes (p≤ 0.01). Combination of the MC₃ agonist with TNF-α caused the highest increase in HO-1 (2.87 ± 0.12 au, p≤ 0.001) protein with a ~ 7-fold increase compared to media-treated samples, and a ~ 3-fold increase, compared to TNF-α stimulated samples. The expression of α-Tubulin standards was found to be consistent with all treatments. HO-1 production was normalized to the standard expression in that reaction (Figure 3.35 A).
Figure 3.35. Effect of α-MSH and D[TRP]8-γ-MSH on HO-1 protein levels in human C-20/A4 chondrocytic cell line.

Cultures of C-20/A4 chondrocytes were treated for 6 h with: Lane 1: DMEM medium, Lane 2: TNF-α (60 pg/ml), Lane 3: α-MSH (3.0 µg/ml), Lane 4: D[TRP]8-γ-MSH (3.0 µg/ml), Lane 5: α-MSH + TNF-α (60 pg/ml), Lane 6: D[TRP]8-γ-MSH (3.0 µg/ml) + TNF-α (60.0 pg/ml). Bands with sizes corresponding to HO-1 (32 kDa) and α-tubulin (55 kDa) were detected by Western blotting. The image is representative of four individual experiments (Panel A). Comparison of densitometrically quantified HO-1 expression by human C-20/A4 cell-lines is shown in arbitrary units (Panel B). Data are presented as Mean ± SEM of n=4 experiments. †p ≤ 0.01, **p ≤ 0.01, ***p ≤ 0.001 vs. cultures treated with TNF-α alone.
3.3.6 Effect of melanocortin peptides on chondrocyte apoptosis.

3.3.6.1 Effect of \( \alpha \)-MSH and D[TRP]\(^8\)-\( \gamma \)-MSH on basal caspase 3/7 activity

Substantial evidence supports that chondrocyte death, due to inflammation and/or injury is an important risk factor predisposing to osteoarthritis and one that exacerbates the disease progression, as chondrocytes are unable to reproduce and compensate for the lost cells (Sharif et al., 2004; Lopez-Armada et al., 2006). We have shown that melanocortins do not cause any statistically significant increase in the production of pro-inflammatory cytokines from C-20/A4 (Table 3.3), and in order to investigate their effect on chondrocyte apoptosis, we employed Caspase-Glo®-3/7 assay to measure any changes in caspase-3/7 activity following treatment.

C-20/A4 chondrocytes were treated with vehicle (DMEM), \( \alpha \)-MSH or D[TRP]\(^8\)-\( \gamma \)-MSH (0.1 – 30.0 µg/ml) for 6 h and caspase-3/7 activity was determined according to the manufacturer’s instructions (Promega UK Ltd., UK). Both \( \alpha \)-MSH and D[TRP]\(^8\)-\( \gamma \)-MSH (0.1 – 30.0 µg/ml), were did not activate caspase-3/7 apoptotic pathway (Figure 3.36), however \( \alpha \)-MSH caused a moderate non-significant inhibition of the basal level of activity of the enzymes.
Figure 3.36. The effect of α-MSH and D[TRP]²-γ-MSH on caspase 3/7 activity of untreated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were plated at 2.0 x 10⁴ cells/well, and treated with DMEM or D[TRP]²-γ-MSH (0.1 – 30.0 µg/ml) for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Caspase-3/7 activity was determined by Caspase-3/7 Glo Assay (Promega, UK). Dotted line symbolizes Caspase 3/7 activity following PBS treatment (control). Data are presented as Mean ± SEM of n=4 experiments, assessed in triplicates.
3.3.6.2 Melanocortin peptides inhibit caspase-3 production, caspase-3/7 activity and cell death of TNF-α-activated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were treated with TNF-α (60.0 pg/ml) to establish levels of caspase-3 activity (Figure 3.37). Western blot analysis against cleaved (activated) caspase-3 (Asp-175) showed 0.52 ± 0.045 au basal production (DMEM) of cleaved caspase-3, which upon stimulation with TNF-α led to 0.65 ± 0.24 au thereby representing a significant 24 % increase in production ($p \leq 0.05$).

Upon pre-treatment of the cells with MC pan-agonist α-MSH and the selective MC$_3$ agonist D[TRP]$^8$-γ-MSH, there was significant reduction in the synthesis of activated caspase-3 compared to the levels caused by TNF-α, with a 50 % (0.32 ± 0.012 au) and 42 % (0.37 ± 0.03) reduction for α-MSH (3.0 µg/ml) and D[TRP]$^8$-γ-MSH (3.0 µg/ml) respectively ($p \leq 0.01$).

In order to investigate the involvement of the specific melanocortin receptors, SHU9119 (10.0 µg/ml) was utilized to specifically block MC$_3$. When used in conjunction with α-MSH it did not block the effect of the peptide however, the antagonist did cause a slight (~8.7 %; n.s. $p>0.05$) synergistic inhibition in the production of activated caspase-3 (0.27 ± 0.02 au) compared to the effect of α-MSH + TNF-α alone.

The opposite effect was observed when SHU9119 was given in conjunction with the selective MC$_3$ agonist D[TRP]$^8$-γ-MSH, whereby it markedly hindered D[TRP]$^8$-γ-MSH from activating its receptor (Figure 3.37). The combination led to significant ~25 % increase in cleaved caspase-3 production (0.47 ± 0.01 au; $p \leq 0.01$) compared to the effect of D[TRP]$^8$-γ-MSH without the antagonist (0.37 ± 0.3 au).

Cell viability was evaluated using MTT proliferation assay (Figures 3.38, 3.39). C-20/A4 chondrocytes were treated with DMEM, α-MSH or D[TRP]$^8$-γ-MSH (0.1 – 30.0 µg/ml) ± TNF-α (60.0 pg/ml) for 6 h with cell viability determined via MTT assay and caspase-3/7 activity determined by Caspase-3/7® Glo Assay. TNF-α (60.0 pg/ml) caused a 26 % cell death as determined by MTT Assay, which was alleviated in a concentration-dependent manner by treatment with the peptides;
maximal inhibition of cell death was achieved at 1.0 and 3.0 µg/ml of α-MSH, and 3.0 and 10.0 µg/ml D[TRP]^8-γ-MSH (p ≤ 0.01).

Treatment of the C-20/A4 chondrocytes with TNF-α increased caspase-3/7 activity 5.7-fold compared to DMEM, but was attenuated in the presence of α-MSH and D[TRP]^8-γ-MSH in a concentration-dependent manner (p ≤ 0.01; Figure 3.38). Pre-treatment of cells with the MC₃/₄ antagonist SHU9119 fully reversed the effect of D[TRP]^8-γ-MSH, but had no effect on α-MSH effect on these parameters.
Figure 3.37. Western Blot detection and densitometric quantification of Cleaved caspase-3 protein levels in human C-20/A4 chondrocytic cell line.

Cultures of C-20/A4 chondrocytes were treated for 6 h with: Lane 1: DMEM medium, Lane 2: TNF-α (60 pg/ml), Lane 3: α-MSH (3.0 µg/ml), Lane 4: D[TRP]8-γ-MSH (3.0 µg/ml), Lane 5: α-MSH + TNF-α (60 pg/ml), Lane 6: D[TRP]8-γ-MSH (3.0 µg/ml) + TNF-α (60 pg/ml). Bands with sizes corresponding to Cleaved caspase-3 (Asp175; 17, 19 kDa) and α-tubulin (55 kDa) were detected by Western blotting. The image is representative of 4 individual experiments (Panel A). Comparison of densitometrically quantified Cleaved caspase-3 (Asp175) expression by human C-20/A4 cell-lines is shown in arbitrary units (au, Panel B). Data are presented as Mean ± SEM of n=4 experiments: **p≤ 0.01, ***p≤ 0.001 vs. respected control.
Figure 3.38. The effect of α-MSH on cell viability and caspase 3/7 activity on TNF-α-activated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were plated at 2.0 x 10^4 cells/well, and treated with PBS or α-MSH (0.1 – 30.0 µg/ml) for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell viability was determined via MTT reduction assay (Panel B) and caspase-3/7 activity determined by Caspase-3/7 Glo Assay (Panels A and B). Dashed (red) line represents control sample cell viability – untreated cells as determined by MTT (100%); Dotted line symbolizes Caspase 3/7 activity following DMEM treatment (control). Data are presented as Mean ± SEM of n=4 experiments, assessed in triplicate. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.01 vs. TNF-α-treated controls; †p ≤ 0.01 vs. untreated controls.
Figure 3.39. The effect of D[TRP]8-γ-MSH on cell viability and caspase 3/7 activity on TNF-α-activated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were plated at 2.0 x 10^4 cells/well, and treated with PBS or D[TRP]8-γ-MSH (0.1 – 30.0 µg/ml) for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell viability was determined via MTT reduction assay (Panel A) and caspase-3/7 activity determined by Caspase-3/7 Glo Assay (Panel A and B). Dashed (red) line represents control sample cell viability – untreated cells (100%); Dotted line symbolizes Caspase 3/7 activity following PBS treatment (control). Data are presented as Mean ± SEM of n=4 experiments, assessed in triplicate. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 vs. vs. TNF-α-treated controls; †p ≤ 0.01 vs. untreated controls.
Figure 3.40. The effect of α-MSH and D[TRP]8-γ-MSH on cell viability following TNF-α activation.

C-20/A4 chondrocytes were plated at 2.0 x 10⁴ cells/well, and pre-treated with α-MSH/D[TRP]8-γ-MSH (0.1 – 30.0 µg/ml) ± SHU9119 (10.0 µg/ml) for 30 min prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell viability was determined via MTT reduction assay. Dashed line represents TNF-α (60.0 pg/ml) treated control cell viability – 74.13%. Data are presented as Mean ± SEM of n=4 experiments, assessed in triplicate. *p≤ 0.05, **p≤ 0.01, compared to respective control.
3.3.7 Effect of hypo-osmolarity on C-20/A4 chondrocyte function.

Cartilage hydration plays an important role in the physiology of healthy chondrocytes. Highly hydrated proteoglycans inflate the cartilage tissue and thus it confers an ability to resist compressive forces (Hall, 1998). However, during osteoarthritis, matrix metalloproteinases are significantly over-expressed, driving the major degradation of collagen that occurs during OA (Kevorkian et al., 2004, Kobayashi et al., 2005). The damaged collagen network allows the charged proteoglycans to attract more water, which leads to dilution of the charged ions in the extracellular space and misbalance of osmolarity compared to the chondrocytic cytoplasm, thereby increased water-flow into the cell and altered metabolism (Maroudas, 1976, Bush and Hall, 2001a, Bush and Hall, 2005). As it is very difficult to mimic these conditions in situ and in vivo, the effect of hypotonic media (140 mOsm:H2O) on chondrocyte function was investigated in C-20/A4 chondrocyte system. The production of pro-inflammatory cytokine production and expression of matrix metalloproteinases was investigated following treatment of the chondrocytes with hypo-osmotic (140 mOsm) DMEM culture media.

3.3.7.1 Effect of hypo-osmolarity on pro-inflammatory cytokines release and MMPs expression in C-20/A4 chondrocytes.

C-20/A4 chondrocytes stimulated with TNF-α (60 pg/ml) showed increased pro-inflammatory cytokine expression and release (IL-1β, IL-6, IL-8) and significant rise in MMP1 and MMP13. Here, an in vitro model, was designed to mimic the hypo-osmolarity in osteoarthritic cartilage in order to investigate its effects on the production of the catabolic pro-inflammatory cytokines IL-6 and IL-8, and the detrimental collagenases 1 and 3.

C-20/A4 chondrocytes were grown in hypotonic media (140 mOsm) for 0, 24 and 72 h and IL6 and IL8 expression determined using semi-quantitative PCR amplification reactions with the appropriate primers (Table 2.9). PCR products were visualised on 2 % agarose gel and the results were normalised to internal control β-actin (Figure 3.41 A). The resulting bands were analysed by densitometry (Figure 3.41 B). There was a significant increase in IL6 and IL8.
mRNA levels. Cells incubated for 24 h in 140 mOsm DMEM media released 37-fold more IL6 compared to control cultures and with increasing the time-periods to 72 h, the expression of IL6 increased to 42-fold compared to cells incubated in normal 280 mOsm DMEM (Figure 3.41). Similarly, IL8 expression was significantly increased following incubation with hypo-osmotic solution. The treatment of C-20/A4 cells with hypo-osmotic solution for 24 h caused a 8-fold up-regulation of IL8 expression, which continued to increase to 10-fold at 72 h post-stimulation.

Changes in protein levels of IL-6 and IL-8 were investigated following incubation of the cells in 140 mOsm DMEM for 24 and 72 h by ELISA (Figure 3.42). C-20/A4 cells were incubated with 140 mOsm DMEM media for 24 or 72 h. Cell-free supernatants were collected and analysed for IL-6 and IL-8 production by commercially available IL-6 and IL-8 ELISAs according to manufacturer’s instructions (R&D Systems, UK). There was a significant time-dependent increase in the concentration of IL-6 as compared to time 0. C-20/A4 chondrocytes responded to decreased osmolarity of the extracellular space (DMEM) with a release of 150 ± 12.66 pg/ml and 192.34 ± 7.83 pg/ml of IL-6 following incubation with the hypo-osmotic medium for 24 and 72h respectively (p≤ 0.001 for both values) compared to time 0 (21.09 ± 18.5 pg/ml). Similar observations were made for IL-8 production following incubation with 140 mOsm DMEM media for 24 and 72h, which yielded 394.32 ± 15.60 pg/ml and 376.10 ± 11.38 pg/ml respectively (p≤ 0.001), compared to the untreated control cells (72.65 ± 4.78 pg/ml). There was slight but insignificant reduction in IL-8 production between 24 and 72 h post stimulation (Figure 3.42).
Figure 3.41. Densitometric quantification of IL-6 and IL-8 expression in C-20/A4 cells incubated in hypotonic (140 mOsm) DMEM media for 24 h and 72 h.

C-20/A4 chondrocytes were stimulated with hypotonic media (140 mOsm) at time 0 and total RNA extracted at 0 h, 24 h and 72 h post stimulation. PCR amplification with the respective primers for IL6 and IL8 was used to detect and quantify gene expression on 2% agarose gels in triplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified IL-6 (Panel B) and IL-8 (Panel C) shown in arbitrary units, each value normalized to the respective β-actin expression. Data are presented as Mean ± SEM of n=4 independent experiments ***p≤ 0.001).
Figure 3.42 Time dependent effect of hypo-osmotic (140 mOsm) DMEM media on IL-6 and IL-8 protein release.

C-20/A4 chondrocytes were treated with hypotonic media (140 mOsm) at time 0 and cell-free supernatants were collected at 0, 24 and 72 h post stimulation. Supernatants were then analysed by commercially available ELISA for IL-6 and IL8 production. Data are presented as Mean ± SEM of n=3 independent experiments repeated in triplicate, **p ≤ 0.01, ***p ≤ 0.001) vs. Time 0.

The effect of hypotonic media on MMP1, MMP3 and MMP13 expression was determined using PCR amplification reactions with the appropriate primers (Table 2.9). The PCR products were visualised on 2 % agarose gel and the results were normalised to internal control β-actin (Figure 3.43 A). Densitometric analysis showed that upon incubation of C-20/A4 chondrocytes with 140 mOsm DMEM media for 24 h and 72 h, MMP1 expression was significantly elevated with 0.49 ± 0.12 au (2.3-fold increase; p ≤ 0.05) at 24 h and a 4-fold increase at 72 h (0.932 ± 0.0263 au; p ≤ 0.001 vs. Time 0 h). Interestingly, MMP3 expression was increased slightly following 72 h compared to non-treated cells (0.714 ± 0.04 au, 1.23-fold; p ≤ 0.05 vs. Time 0), whilst no significant changes were observed at 24 h. MMP13, a collagenase that is expressed in neither non-stimulated C-20/A4 cells, (as shown on Figure 3.43 B), nor in healthy human cartilage, was significantly up-regulated to 0.557 ± 0.058 au after 24 h incubation in 140 mOsm DMEM (p ≤ 0.001), and expression was increased following 72 h (0.642 ± 0.01 au; p ≤ 0.001 vs. Time 0).
Figure 3.43. Densitometric quantification of MMP1, MMP3 and MMP13 expression in C20/A4 cells treated with 140 mOsm DMEM media.

C20/A4 chondrocytes were stimulated with hypotonic media (140 mOsm) at time 0 and total RNA extracted 0-72 h post stimulation. PCR amplification with the respective primers for MMP1, MMP3 and MMP13 was used to detect and quantify gene expression on 2 % agarose gels in duplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified MMP1, MMP3 and MMP13 expression induced by hypotonic saline (140 mOsm:H2O, Panel B) shown in arbitrary units, each value normalized to the respective β-actin expression. Data is presented as Mean± SEM of n=4 independent experiments *p≤ 0.05, ***p≤ 0.001).
3.3.7.2 Attenuation of extracellular hypo-osmolarity-induced matrix metalloproteinases production by melanocortin peptides α-MSH and D[TRP]8-γ-MSH.

In order to investigate different aspects of the anti-inflammatory properties of the melanocortin peptides α-MSH and D[TRP]8-γ-MSH, their effect was evaluated on the up-regulated expression of catabolic proteinases caused by the hypo-osmotic challenge.

C-20/A4 cells were incubated in 140 mOsm DMEM for 24 h + α-MSH (3.0 µg/ml) or D[TRP]8-γ-MSH (3.0 µg/ml). Total RNA was extracted and MMP1, MMP3 and MMP13 gene expression was analysed by semi-quantitative PCR (Figure 3.44 A) with the specific primers for the amplification of the individual genes listed in Table 2.9. Following treatment for 24 h, α-MSH failed to cause any significant inhibition of the expression of MMP1, MMP3 and MMP13. Similarly, D[TRP]8-γ-MSH at 3.0 µg/ml failed to cause any detectable change in the expression of MMP3, however, the MC3 agonist reduced the transcription of MMP1 (from 0.49 ± 0.12 au to 0.25 ± 0.05 au) and MMP13 (from 0.56 ± 0.06 au to 0.11 ± 0.01 au), representing 49 % and 80 % inhibition respectively (Figure 3.44 B).
Figure 3.44. Effect of melanocortins on MMP1, MMP3 and MMP13 expression in C-20/A4 cells treated with 140 mOsm DMEM for 24 h measured by densitometry.

C-20/A4 chondrocytes were pre-treated with α-MSH or D[TRP]8-γ-MSH prior to stimulated with hypotonic media (140 mOsm) at time 0 and total RNA extracted 24 h post stimulation. PCR amplification with the respective primers for MMP1, MMP3 and MMP13 was used to detect and quantify gene expression on 2 % agarose gels in duplicates with β-actin used as internal control (Panel A). Comparison of densitometrically quantified MMP1, MMP3 and MMP13 expression induced by hypotonic saline (140 mOsm; Panel B) shown in arbitrary units, each value normalized to the respective β-actin expression. Data are presented as Mean ± SEM of n=3 independent experiments *p≤ 0.05).
3.4 Effect of pro-inflammatory stimuli on primary bovine chondrocytes function.

3.4.1 Detection of pro-inflammatory cytokines release following primary chondrocyte activation.

C-20/A4 cell-line chondrocytes have been shown within this thesis to respond to TNF-α and LPS stimulation leading to the release of pro-inflammatory cytokines. Given these findings, primary bovine articular chondrocytes (freshly extracted, cultured in monolayers) were evaluated to determine whether primary cells responded in a similar fashion, as these have been shown to be a good model for human primary chondrocytes (Kerrigan and Hall, 2005). Isolated bovine articular chondrocytes were cultured and incubated in serum-free 380 mOsm DMEM medium for 24 h prior to stimulation with TNF-α (20.0 – 60.0 pg/ml) and LPS (1.0 – 10.0 µg/ml) for 6 h, cell-free supernatants were collected and analysed for IL-1β, IL-6 and IL-8 by ELISA.

3.4.1.1 Effect of TNF-α and LPS on IL-6 and IL-8 release from bovine chondrocytes.

TNF-α stimulation of primary bovine chondrocytes led to significant increases in IL-6 release (Figure 3.45 A). The pro-inflammatory cytokine (20.0 – 80.0 pg/ml) caused a concentration-dependent increase in synthesis and release of IL-6 from the bovine articular chondrocytes with 20.0 pg/ml causing a significant release of 38.54 ± 9.85 pg/ml of IL-6 (p≤ 0.05) compared to untreated controls (3.2 ± 1.38 pg/ml). IL-6 levels doubled following stimulation with 40.0 pg/ml (78.59 ± 13.68 pg/ml, p≤ 0.01). Increasing concentrations of TNF-α led to 174.38 ± 14.6 pg/ml (p≤ 0.001) and 347.07 ± 29.79 pg/ml (p≤ 0.001) of IL-6 production at 60.0 pg/ml and 80.0 pg/ml respectively. LPS (1.0 – 10.0 µg/ml) also caused significant increases in IL-6; however, all concentrations evaluated caused a similar response. A maximal response was achieved following stimulation with LPS (3.0 µg/ml), where 163.23 ± 12.24 pg/ml of IL-6 was released. Both 1.0 and 10.0 µg/ml of LPS had similar effect, causing 156.93 ± 15.35 pg/ml and 159.12 ± 12.24 pg/ml of IL-6 release respectively. The increase caused by LPS at all
concentrations was significantly increased compared to the untreated controls (3.2 ± 1.38 pg/ml; p≤ 0.001; Figure 3.45 B).

Following the determination of IL-6, IL-8 levels from stimulated bovine chondrocytes were evaluated. TNF-α (20.0 – 80.0 pg/ml) caused a bell-shaped concentration dependent effect, peaking at 40.0 pg/ml and 60.0 pg/ml with 46.67 ± 13.65 pg/ml (p<0.05) and 45.56 ± 2.94 pg/ml (p≤ 0.01) respectively, whilst higher concentrations of TNF-α (80.0 pg/ml) caused lower levels of IL-8 release (28.89 ± 3.9 pg/ml (p≤ 0.01; Figure 3.45 C). LPS (1.0 – 10.0 µg/ml) caused a similar response with 22.33 ± 3.1 pg/ml (p≤ 0.01) and 12.29 ± 1.98 pg/ml (p≤ 0.05) IL-8 release for 3.0 µg/ml and 10.0 µg/ml respectively, however, 1.0 µg/ml of LPS failed to induce any significant increase in IL-8 compared to control values (p> 0.05; Figure 3.45 B).

3.4.1.2 Effect of TNF-α and LPS on IL-1β and MCP-1 release from bovine chondrocytes.

Treatment of bovine chondrocytes with TNF-α (20.0 – 80.0 pg/ml) caused a bell shaped concentration-dependent effect on IL-1β release following 6 h incubation, peaking at 60.0 pg/ml with 21.87 ± 0.2 pg/ml (p≤ 0.05) compared to untreated controls (0.82 ± 0.06 pg/ml; Figure 3.46 A). Lower concentrations of TNF-α caused 17.06 ± 0.65 pg/ml and 17.40 ± 0.23 pg/ml IL-1β release for 20.0 and 40.0 pg/ml respectively (p≤ 0.05). Similar response was observed following stimulation with the highest tested concentration 80.0 pg/ml of TNF-α leading to 17.92 ± 0.26 pg/ml IL-1β released (p≤ 0.05). LPS stimulation also activated the bovine chondrocytes to produce IL-1β at all concentrations tested (Figure 3.46 B), with a maximal release following stimulation with 3.0 µg/ml, causing a release of 20.8 ± 3.8 pg/ml IL-1β (p≤ 0.05). LPS at 1.0 and 10.0 µg/ml led to significant 17.9 ± 1.2 pg/ml and 19.8 ± 2.9 pg/ml IL-1β release from the chondrocytes compared to control (p≤ 0.05).

Similarly to the effect on IL-1β, IL-6 and IL-8, TNF-α (20.0 – 80.0 pg/ml) and LPS 1.0 – 10.0 µg/ml) caused significant production of MCP-1 (Figure 3.46 C). TNF-α caused a maximal response at 60.0 pg/ml with 159.44 ± 13.29 pg/ml (p≤ 0.001), whilst 20.0 pg/ml was unable to exhibit any effect on the production of MCP-1,
but when the concentration was doubled to 40.0 pg/ml, there was a significant elevation in MCP-1 (79.86 ± 25.12 pg/ml; \( p \leq 0.001 \)) compared to untreated controls.

When primary bovine cells were treated for 6 h with LPS (1.0 – 10.0 \( \mu \)g/ml) there was a significant increase in MCP-1, with a maximal production of 108.05 ± 4.2 pg/ml at 3.0 \( \mu \)g/ml LPS. All other concentrations caused very similar amount of MCP-1 production (\( p \leq 0.001 \) for all doses of LPS used; Figure 3.46 D).
Figure 3.45  Effect of TNF-α and LPS on IL-6 and IL-8 release from extracted bovine chondrocytes.

Bovine chondrocytes were extracted from cartilage explants and cultured for plated for 24 h in serum-free 380 mOsm DMEM prior to stimulation. Panel A and C: TNF-α (20.0-80.0 pg/ml) and Panel B and D: LPS (1.0-3.0 µg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-6 IL-8 levels by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments performed in triplicate. *p ≤ 0.05, **p ≤ 0.01 vs. untreated samples (0.0).
Figure 3.46  Effect of TNF-α and LPS on IL-1β and MCP-1 release from extracted bovine chondrocytes.

Bovine chondrocytes were extracted from cartilage explants and cultured for plated for 24 h in serum-free 380 mOsm DMEM prior to stimulation. Panel A and C: TNF-α (20.0 – 80.0 pg/ml) and Panel B and D: LPS (1.0 – 3.0 µg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-1β (Panels A, B) and MCP-1 (Panels C, D) levels by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments performed in triplicate. **p ≤ 0.01, ***p ≤ 0.001) vs. untreated controls (0.0).
3.4.2 Melanocortin receptors expression in bovine articular chondrocytes.

Following the identification of cytokine release from primary bovine chondrocytes, expression of MC was determined. Gene and species specific primers were designed for both \( \text{MC}_1 \) and \( \text{MC}_5 \) in order to determine whether \( \text{MC}_1 \) and \( \text{MC}_5 \) were expressed in the articular chondrocytes of *Bos taurus*. However, the full coding sequence of \( \text{MC}_3 \) has not been revealed yet, therefore it was not possible to investigate its expression at that point. Total mRNA was extracted from non-stimulated chondrocytes extracted from at least 5 individual bovine joints and conventional RT-PCR used to quantify the transcriptional levels of both genes.

There was a marked expression of \( \text{MC}_1 \) that was consistent between the individual animals and confirmed by the detection of size-specific bands on 2 % agarose gels, corresponding to the expected size of \( \text{MC}_1 \) fragment of interest (Figure 3.47). The transcriptional studies of \( \text{MC}_5 \) were non-conclusive, since the expression of the gene varied significantly between the tested animal tissues. All samples were run alongside GAPDH internal control.

![Figure 3.47 Endogenous expression of MC1 and MC5 in primary bovine chondrocytes.](image)

Freshly extracted bovine primary chondrocytes were cultured for 24 h in serum-free 380 mOsm DMEM media and total RNA was subsequently extracted. PCR amplification with the respective primers for bovine \( \text{MC}_1 \) and \( \text{MC}_5 \) were used to detect and quantify gene expression on 2% agarose gels in triplicates with *Gapdh* used as internal control. Densitometrically quantified \( \text{MC}_1 \) and \( \text{MC}_5 \) shown in arbitrary units, each value normalized to the respective GAPDH expression. Data is presented as Mean ± SEM of cartilage from 5 different animals.
3.4.3 Effect of melanocortin peptides on pro-inflammatory cytokine release from TNF-α stimulated primary bovine chondrocytes.

Since the coding region of MC3 was not available to design the primers needed for the investigative study of MC3 expression, another approach was used that allowed research into whether MC3 was expressed by using the selective MC3 agonist [DTRP$^8$]-γ-MSH. The pan-agonist α-MSH, MC3 agonist D[TRP]$^8$-γ-MSH and MC3/4 antagonist/MC1 agonist SHU9119 were used in separate experiments to reveal whether MC1 and MC3 were present and able to modulate the release of pro-inflammatory mediator release from primary bovine articular chondrocytes.

In order to investigate the validity and efficacy of melanocortin peptides at inhibiting the release of pro-inflammatory cytokines following TNF-α stimulation, they were investigated in primary bovine chondrocytes (Figure 3.48). Primary bovine chondrocytes were extracted from bovine cartilage and monolayer cultures incubated in serum-free 380 mOsm DMEM medium for 24 h prior to pre-treatment for 30 min with α-MSH, D[TRP]$^8$-γ-MSH or SHU9119 (1.0 – 30.0 µg/ml) prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Pre-treatment of primary bovine chondrocytes with α-MSH, D[TRP]$^8$-γ-MSH and SHU9119 caused a significant decrease in IL-β levels. Concentrations between 1.0 and 10.0 µg/ml of α-MSH significantly reduced the levels of IL-1β produced by TNF-α from 21.87 ± 0.23 pg/ml down to 12.85 ± 0.29 pg/ml (α-MSH 1.0 µg/ml), representing a 41% decrease (p ≤ 0.05). α-MSH (3.0 µg/ml) decreased IL-1β concentration to 13.30 ± 0.71 pg/ml, a 39 % reduction (p ≤ 0.05). Increasing the peptide concentration did not alter the inhibition of IL-1β compared to the lower 3.0 µg/ml concentration with a similar 39 % reduction. Higher concentrations of α-MSH (30.0 µg/ml) did not have a statistically significant effect (17.34 ± 1.4 pg/ml; p > 0.05; Figure 3.48) compared to TNF-α–treated controls.

Treatment of primary chondrocytes with D[TRP]$^8$-γ-MSH caused a bell shaped concentration dependent decrease in IL-1β levels with 1.0 µg/ml being essentially inactive (19.68 ± 1.58 pg/ml; p > 0.05). Levels of IL-1β were reduced by 61 % following stimulation with 3.0 µg/ml (p ≤ 0.05) and a maximal effect of 76 % at 10.0 µg/ml (p ≤ 0.05), and starting to slowly decrease efficacy when the
concentration was increased to 30.0 µg/ml (39%). A modest inhibitory effect was observed with SHU9119 with 1.0 µg/ml (16.84±0.72 pg/ml; \( p \leq 0.05 \)) and 3.0 µg/ml (17.46 ± 0.9 pg/ml; \( p \leq 0.05 \)) causing a reduction of 23 % and 20 % respectively. Higher concentrations had no significant effect on TNF-\( \alpha \) induced IL-1\( \beta \) levels (Figure 3.48).

![Figure 3.48 Effect of melanocortin peptides on IL-1\( \beta \) release from TNF-\( \alpha \) activated primary bovine chondrocytes.](image)

Bovine chondrocytes were extracted from bovine cartilage and cultured for 24 h in serum-free 380 mOsm DMEM medium. Chondrocytes pre-treated with \( \alpha \)-MSH, D[TRP]\(^8\)-\( \gamma \)-MSH or SHU9119 (1.0-30.0 µg/ml) for 30 mins, prior to stimulation with TNF-\( \alpha \) (60.0 pg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-1\( \beta \) levels by ELISA. Data are presented as Mean\( \pm \) SEM of \( n=4 \) independent experiments repeated in triplicate, \(* p \leq 0.05 \) vs. culture treated with TNF-\( \alpha \) alone. Dashed line represents TNF-\( \alpha \) treatment; dotted line – untreated cultures.
α-MSH caused a significant abrogation of TNF-α-induced IL-6 release from primary bovine chondrocytes (Figure 3.49 A). TNF-α-stimulation alone caused 174.38 ± 14.6 pg/ml of IL-6 release. α-MSH was most effective at 1.0 µg/ml causing a 92.7 % inhibition in IL-6 release (12.74 ± 1.2 pg/ml; p ≤ 0.001). Higher concentrations of peptide still exerted a significant inhibitory effect with 3.0 µg/ml causing an 87.3 % decrease (22.18 ± 0.9 pg/ml; p ≤ 0.001), 10.0 µg/ml causing a 76.4 % reduction (41.22 ± 0.57 pg/ml; p ≤ 0.001) and 30.0 µg/ml a 48% decrease (90.8 ± 10.24 pg/ml; p ≤ 0.01) compared to control.

Following identification of the inhibitory effect of α-MSH, the MC₃ agonist D[TRP]⁸-γ-MSH was evaluated. D[TRP]⁸-γ-MSH (1.0 – 10.0 µg/ml) caused a bell shaped inhibitory effect of IL-6 release (Figure 3.49 A). The maximal effect was observed at 3.0 µg/ml with a 72 % decrease in IL-6 release, whilst 1.0 and 10.0 µg/ml had a reduced inhibitory effect displaying 40.1 % (104.46 ± 3.11 pg/ml; p ≤ 0.01) and 48.2 % (90.34 ± 16.29 pg/ml; p ≤ 0.01) reduction respectively. Higher concentrations (30.0 µg/ml) did not cause a statistically significant change in the release of IL-6 (p > 0.05) compared to TNF-α alone. The MC₃/4 antagonist/MC₁ agonist SHU9119 failed to inhibit IL-6 release at all concentrations evaluated (Figure 3.49 A).

D[TRP]⁸-γ-MSH attenuated IL-8 release elicited by TNF-α (60.0 pg/ml) in a bell shaped dependent fashion (Figure 3.49 B). The maximal effect was reached at 3.0 µg/ml causing a 69.4 % decrease in IL-8 levels (13.93 ± 1.13 pg/ml; p ≤ 0.01) compared to TNF-α treated controls (45.56 ± 2.94 pg/ml), a similar reduction was observed at 1.0 µg/ml with a 66.5 % decrease. At 10.0 µg/ml a 57 % inhibition was observed, however at 1.0 and 30.0 µg/ml of D[TRP]⁸-γ-MSH failed to inhibit IL-8 release (p > 0.05). In contrast all concentrations of α-MSH evaluated caused a significant inhibition of IL-8 with a maximal inhibition being observed at 3.0 µg/ml of 66.02 % (15.49±1.28 pg/ml). As previously observed with IL-6, SHU9119 failed to inhibit significantly inhibit IL-8 release at all concentrations evaluated (p ≤ 0.05; Figure 3.49 B).
Figure 3.49 Effect of melanocortin peptides on IL-6 and IL-8 release from TNF-α-activated primary bovine chondrocytes.

Bovine chondrocytes were extracted from bovine cartilage and cultured for 24 h in serum-free 380 mOsm DMEM medium. Chondrocytes pre-treated with α-MSH, D[TRP]8-γ-MSH or SHU9119 (1.0-30.0 µg/ml) for 30 mins, prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-6 and IL-8 levels by ELISA. Data is presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 vs. TNF-α alone.
3.4.4 Effect of MC3/4 antagonist SHU9119 on anti-inflammatory effect of α-MSH and D[TRP]8-γ-MSH in TNF-α activated bovine primary chondrocytes.

To try and unravel whether MC1 and/or MC3 was mediating the effects of the melanocortin peptides α-MSH and D[TRP]8-γ-MSH, SHU9119 was added on TNF-α activated primary P0 bovine articular chondrocytes. Primary P0 bovine articular chondrocytes were extracted and cultured in a monolayer with serum-free 380 mOsm DMEM medium for 24 h prior to stimulation. The cells were treated with the peptides (1.0 – 10.0 µg/ml) for 30 min ± SHU9119 (10.0 µg/ml) prior to stimulation with TNF-α (60.0 pg/ml) for 6 h and IL-1β, IL-6 and IL-8 levels were detected by ELISA (Figure 3.50). SHU9119 failed to significantly block the effect of α-MSH at 1.0 and 3.0 µg/ml (p>0.05), surprisingly however, at 10.0 µg/ml α-MSH the inhibitory effect on IL-1β was blocked in the presence of SHU9119 (Figure 3.50 A). In contrast SHU9119 (10.0 µg/ml) abrogated the anti-inflammatory effect of D[TRP]8-γ-MSH at all concentrations evaluated (Figure 3.50 B).

α-MSH inhibitory effect on IL-6 was not blocked in the presence of SHU9119 at 1.0 and 3.0 µg/ml. (Figure 3.51 A) However when SHU9119 (10.0 µg/ml) was combined with higher doses of α-MSH (10.0 µg/ml), there was a synergistic effect that caused a marked decrease in IL-6 levels (13.33 ± 3.22 pg/ml) a 66.7 % reduction compared to α-MSH alone (41.22 ± 0.56 pg/ml). In contrast D[TRP]8-γ-MSH at all concentrations evaluated (1.0 – 10.0 µg/ml) was completely blocked when SHU9119 was given in combination (Figure. 3.51 B). IL-8 released following TNF-α stimulation of P0 chondrocytes showed that SHU9119 (10.0 µg/ml) failed to inhibit the anti-cytokine effects of α-MSH (1.0 – 10.0 µg/ml, Figure 3.51 C). D[TRP]8-γ-MSH inhibition of IL-8 was abrogated in the presence of SHU9119 (Figure 3.51 D).

Finally, given that SHU9119 blocked the action of D[TRP]8-γ-MSH but not of α-MSH, we evaluated the effect of this combination on IL-10 synthesis. Treatment of the P0 bovine chondrocytes with α-MSH led to significant increase in IL-10 levels (Figure 3.52 A), there was no synergistic effect when cells were incubated
with a combination of α-MSH and SHU9119 and the peptide’s actions were not significantly affected by the addition of the MC₃/₄ antagonist. There was a general reduction in the melanocortin effect, which could be attributed to its partial binding affinities to MC₃ in contribution to MC₁. However, SHU9119 prevented D[TRP]₈-γ-MSH from binding to its receptor, therefore reduced significantly IL-10 production (Figure 3.52 B). SHU9119 caused 89 % reduction of IL-10 in 1.0 µg/ml and 10.0 µg/ml of D[TRP]₈-γ-MSH treated cells, and 86 % when given simultaneously with 3.0 µg/ml of D[TRP]₈-γ-MSH.
Figure 3.50 Effect of the MC₃/₄ antagonist SHU9119 on IL-1β release from TNF-α activated primary bovine articular chondrocytes following α-MSH and D[TRP]₈-γ-MSH treatment.

Bovine chondrocytes were extracted from bovine cartilage and cultured for 24 h in serum-free 380 mOsm DMEM medium. Chondrocytes were then pre-treated with α-MSH (Panel A) or D[TRP]₈-γ-MSH (1.0 – 10.0 µg/ml; Panel B) ± SHU9119 (10.0 µg/ml) for 30 mins, prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-1β levels by ELISA. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, **p≤0.01 vs. TNF-α alone.
Figure 3.51. Effect of the MC₃/₄ antagonist SHU9119 on IL-6 release from TNF-α activated primary bovine articular chondrocytes following α-MSH and D[TRP]⁸-γ-MSH treatment.

Bovine chondrocytes were extracted from bovine cartilage and cultured for 24 h in serum-free 380 mOsm DMEM medium. Chondrocytes were then pre-treated with α-MSH (Panel A) or D[TRP]⁸-γ-MSH (Panel B; 1.0 – 10.0 µg/ml) ± SHU9119 (10.0 µg/ml) for 30 mins, prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-6 levels by ELISA. Data is presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, **p≤ 0.01 vs. TNF-α alone.
Figure 3.52 Effect of the MC₃/₄ antagonist SHU9119 on IL-10 release from TNF-α activated primary bovine articular chondrocytes following α-MSH and D[TRP]⁸-γ-MSH treatment.

Bovine chondrocytes were extracted from bovine cartilage and cultured for 24 h in serum-free 380 mOsm DMEM medium. Chondrocytes were then pre-treated with α-MSH (Panel A) or D[TRP]⁸-γ-MSH (Panel B; 1.0 – 10.0 µg/ml) ± SHU9119 (10.0 µg/ml) for 30 mins, prior to stimulation with TNF-α (60.0 pg/ml) for 6 h. Cell-free supernatants were collected and analysed for IL-10 levels by ELISA. Data is presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p≤ 0.05, **p≤ 0.01 vs. TNF-α alone.
3.4.5 Cartilage impact and the effect of melanocortins and steroids on bovine chondrocytes in situ.

Mechanical stress above the physiological range can profoundly influence articular cartilage causing matrix damage, changes to chondrocyte metabolism and cell injury/death (Bush and Hall, 2005) and therefore has been implicated as a risk factor for the development of osteoarthritis. The mechanism of cell damage is not understood, but chondrocyte volume could be a determinant of the sensitivity and subsequent response to load (Hall, 1998). In OA, it is possible that the chondrocyte swelling that occurs renders the cells more sensitive to the damaging effects of mechanical stress. Here, changes in viability of in situ chondrocytes following a single blunt impact have been investigated in the presence of α-MSH, D[TRP]8-γ-MSH, and the steroid dexamethasone evaluated at the time of impact and 6h post-impact.

3.4.5.1 Cell viability of articular bovine chondrocytes in vivo following single impact.

Explants of bovine articular cartilage were incubated with the fluorescent indicators 5.0 µM Calcein-AM and 1.0 µM Propidium Iodide, permitting the measurement of cell viability, respectively, using confocal laser scanning microscopy (CLSM). Cartilage was then subjected to a single impact with 1.14 N force delivered from a vertical drop tower, which caused areas of chondrocyte injury/death within the superficial zone (SZ) as shown on Figure 3.52.

Cell death following single impact was quantified using Imaris 7.1.1 Spots analysis software and the results are shown in Figure 3.58. Impact caused a significant (*p<0.05) increase in cell death. Non-impacted cartilage showed 2.95 ± 0.26 % chondrocytes death, which following impact increased to 13.50 ± 1.72% (Figure 3.53). Areas of chondrocyte injury inflicted from impact tower injury can be seen on Figure 3.58 and visually compared to non-impacted counterparts (original 3D volume image reconstitutions [A and B] and spots analysis [a and b] respectively).
Figure. 3.52. A 3D-image of cartilage impacted chondrocytes.
Cartilage explant was incubated for 30 min at 37°C with 5.0 μM Calcein-AM and 1.0 μM Propidium Iodine for staining alive and dead cells respectively and visualized using CLSM at x630 magnification using x63 immersable lens. Iso-surface object were created for all cells, in order to create presentation image of an impacted piece of cartilage (image not used for volume analysis) Red cells represent chondrocytes undergoing cell death, and green – cells that are alive and fully functional at the time of imaging.
Bovine articulate cartilage explants were incubated with the fluorescent indicators Calcein-AM (5.0 µM) and Propidium Iodide (1.0 µM) prior to (Panel A, a) and at 6 h post-mechanical trauma (Panel B, b) for 30 min, and visualized under CLSM. Imaris 7.1.1 Spot Analysis software was used to quantify the number of viable (green) and non-viable (red) cells. Panels a, and b represent Spots analysis of the original CLSM images, taken at x100 magnification (Panels A and B). Percentage cell death was calculated based on the acquired results (Panel C). Data are representative images or Mean ± SEM of n=4 experiments, repeated in triplicates, *p ≤ 0.05 vs. non-impacted control samples.
3.4.5.1.1 The effect of melanocortins and dexamethasone on cell death.

In order to investigate whether the melanocortin peptides have chondroprotective properties, cell viability was quantified as described earlier. Briefly, cells were treated for 6 h with 3.0 µg/ml α-MSH or D[TRP]⁸-γ-MSH, or 1.0 µM dexamethasone and then incubated with the fluorescent indicators Calcein-AM (5.0 µM) and Propidium Iodide (1.0 µM) for 30 min prior to CLSM, permitting the measurement of cell viability.

Figure 3.54 demonstrates that α-MSH caused a reduction in chondrocyte death compared to basal levels (2.95 %). Cell death decreased to 0.97 %, when cartilage explants were incubated for 6 h with α-MSH (3.0 µg/ml) prior to CLSM imaging, representing 67 % reduction in cell death, compared to untreated controls (p≤ 0.05). However, neither D[TRP]⁸-γ-MSH (3.0 µg/ml) nor dexamethasone (1.0 µM) was able to show any significant effect on this parameter.
Figure 3.54. Chondroprotective effect of melanocortin peptides as quantified by Imaris 7.1.1 Spots Analysis software.

Bovine articular cartilage explants were treated with either DMEM (Panel A), α-MSH (3.0 µg/ml; Panel B), D[TRP]<sub>8</sub>-γ-MSH (Panel C), or 1.0 µM dexamethasone (Panel D) for 6 h. Cartilage explants were then incubated for 30 min at 37°C with the fluorescent indicators Calcein-AM (5.0 µM) and Propidium Iodine (1.0 µM) for staining alive and dead cells respectively, immediately following which the explants were visualized using CLSM at x100 magnification. Imaris 7.1.1 Spot Analysis software was used to quantify the number of viable (green) and non-viable (red) cells. Percentage cell death was calculated based on the acquired results (Panel E). Data are representative images or Mean ± SEM of n=4 experiments, repeated in triplicates, *p ≤ 0.05 vs. non-impacted control samples.
3.4.5.1.2 The effect of melanocortins and dexamethasone on pro-inflammatory cytokine release from non-impacted bovine chondrocytes in situ.

In order to determine the basal levels of pro-inflammatory cytokines released from non-impacted bovine cartilage explants, the cartilage pieces were incubated in 380 mOsm DMEM for 24 h, following which the medium was replaced with fresh DMEM and IL-1β, IL-6 and IL-8 release was detected at 6 h by ELISA. Similarly, the effect of the melanocortins on pro-inflammatory cytokine release from non-injured cartilage pieces were evaluated in the supernatants at 6 h, post-treatment of the bovine explants with α-MSH (3.0 µg/ml), D[TRP]³-γ-MSH (3.0 µg/ml) or dexamethasone (1.0 µM) and compared to the basal levels of cytokine release (Figure 3.55).

There was a significant basal production of cytokines detected in the supernatants of non-treated bovine articular cartilage explants, with 17.94 ± 4.61 pg/ml/g (pg/ml per gram of cartilage tested) of IL-1β, 59.33 ± 8.75 pg/ml/g of IL-6 and 97.11 ± 10.57 pg/ml of IL-8, values which were not significantly altered by the addition of any of the drugs as can be seen from Figure 3.55 Panels A, B and C respectively.
Figure 3.55 Effect of α-MSH, D[TRP]^8-γ-MSH and dexamethasone on basal production of IL-1β, IL-6 and IL-8.

Bovine articular cartilage explants were treated with DMEM, α-MSH (3.0 µg/ml), [DTRP]^8-γ-MSH (3.0 µg/ml), or dexamethasone (1.0 µM) for 6 h and cell-free supernatants were collected and analysed for IL-1β (Panel A), IL-6 (Panel B) and IL-8 (Panel C) via commercially available ELISAs (R&D Systems). Results were corrected per gram of tissue tested. Data are presented as Mean ± SEM of n=4 experiments repeated in triplicate. DMEM-treated control samples are represented by a dotted line.
3.4.5.2 The effect of melanocortin peptides and dexamethasone on impacted cartilage explants.

3.4.5.2.1 The effect of melanocortin peptides and dexamethasone on cell death as detected via CLSM.

In order to investigate whether melanocortin peptides were able to exhibit a protective effect on impacted bovine chondrocytes in situ, cell viability was quantified as described previously. Briefly, cells were treated for 30 min with DMEM, α-MSH (3.0 µg/ml), D[TRP]$^8$-γ-MSH (3.0 µg/ml), or dexamethasone (1.0 µM) prior to impact and incubated for 6 h. 30 min prior to CLSM imaging, cartilage explants were incubated with the fluorescent indicators Calcein-AM (5.0 µM) and Propidium iodide (1.0 µM).

All drugs caused a significant decrease in cell death ($p \leq 0.05$) compared to DMEM-treated impacted sample (Figure 3.56). Upon single blunt impact using the vertical drop tower, 13.5 ± 1.72 % of the cells in the injured cartilage were found to be dead 6 h following the impact. Treatment of cartilage explants with melanocortin peptides or dexamethasone for 30 min prior to impact and left to incubate for 6 h resulting in significant protective effects on the chondrocytes. Cell death was reduced to 5.10 ± 1.42 %, when the articular cartilage explants were incubated with α-MSH (3.0 µg/ml), representing a 62% reduction ($p \leq 0.05$). Similar was the effect of the selective MC$_3$ agonist D[TRP]$^8$-γ-MSH with the peptide protecting 66 % of the cells during the first 6 h of impact, allowing for only 4.63 ± 0.62 % of the chondrocytes to undergo cell death ($p \leq 0.05$). Similarly, dexamethasone exhibited chondroprotective properties and caused a 50 % reduction in cell death compared to DMEM-treated impacted cartilage explant with just 6.75 ± 2.38 % of the cells in the injured explant to undergo cell death ($p \leq 0.05$).

3.4.5.2.2 Melanocortin peptides and dexamethasone decrease pro-inflammatory cytokines induced by mechanical trauma.

In order to investigate the involvement of pro-inflammatory cytokines in the chondrocyte function following cartilage injury, bovine articular cartilage explants were subjected to a single impact with a force of 1.12 N. Following the
mechanical trauma, the cartilage were returned to their media for 6 h, when cell-free supernatants were collected. Similarly, the cartilage explants were treated with the melanocortin peptides and dexamethasone for 30 min, following which period they were subjected to single impact and returned back in the same media containing the respective drug for additional 6 h. The supernatants were then collected and analysed for IL-1β, IL-6 and IL-8 production by ELISA.

Upon impact, the chondrocytes in the DMEM-treated impacted explants produced 351.22 ± 24.12 pg/ml/g IL-1β (Figure 3.57), representing a 19-fold increase ($p \leq 0.001$) in the release of this pro-inflammatory cytokine compared to the basal amounts released by the chondrocytes from non-impacted cartilage explants. The injury led to 13-fold increase ($p \leq 0.001$) in IL-6 production compared to basal levels, with 448.41 ± 19.65 pg/ml/g at 6 h post-impact (Figure 3.57 B). Similarly, there was a significant, 3-fold increase in the levels of IL-8 ($p \leq 0.001$), with 293.46 ± 13.94 pg/ml/g (Figure 3.57 C). All of the drugs were found to be effectively reducing these amounts of pro-inflammatory cytokines, produced by the chondrocytes in response to the mechanical trauma. $\alpha$-MSH (3.0 µg/ml) decreased the release of IL-1β by 41 % to 209.14 ± 2.22 pg/ml/g ($p \leq 0.01$) compared to the respective DMEM-treated samples (Figure 3.57 A). The selective MC3 agonist was significantly more potent in inhibiting the release of IL-1β to 175.09 ± 19.13 pg/ml/g (50 %, $p \leq 0.01$). In similar fashion dexamethasone caused an even larger reduction of 58% of IL-1β release (149.80 ± 2.83 pg/ml/g; $p \leq 0.001$). IL-6 release was significantly inhibited in the presence of $\alpha$-MSH, D[TRP]$^8$-γ-MSH and Dexamethasone down to 158.32 ± 12.87 pg/ml/g, 130.88 ± 7.75 pg/ml/g and 73.57 ± 6.26 pg/ml/g for each of the peptides respectively, therefore representing 65%, 71% and 84% reduction in the secretion of this cytokine ($p \leq 0.01$). A similar effect on IL-8 release was observed, with $\alpha$-MSH causing a significant 53 % reduction, with detected levels of 137.26 ± 15.60 pg/ml/g of IL-8 ($p \leq 0.01$, compared to DMEM-treated impacted controls). D[TRP]$^8$-γ-MSH led to 54 % decrease down to 133.81 ± 22.05 ($p \leq 0.01$). Dexamethasone was slightly more effective in reducing IL-8 release with a 65 % decrease (104.10 ± 12.39 pg/ml/g) in the levels, detected in the supernatants of impacted cartilage explant incubated only with DMEM media.
Figure 3.56 Effect of melanocortins and dexamethasone on chondrocyte death following impact as quantified by Imaris 7.1.1 Spots Analysis software.

Bovine articular cartilage explants were treated with either DMEM (Panel A), α-MSH (3.0 µg/ml; Panel B), [DTRP]²γ-MSH (3.0 µg/ml Panel C), or 1.0 µM Dexamethasone (Panel D) for 30 min prior to blunt single impact delivered by a vertical drop tower and incubated for 6 h. Cartilage explants were then incubated for 30 min at 37°C with the fluorescent indicators Calcein-AM (5.0 µM) and Propidium Iodine (1.0 µM) for staining alive and dead cells respectively, immediately following which the explants were visualised using CLSM at x100 magnification. Imaris 7.1.1 Spot Analysis software was used to quantify the number of viable (green) and non-viable (red) cells. Cell death was calculated based on the acquired results (Panel E). Data are representative images or Mean ± SEM of n=4 experiments, repeated in triplicates, *p≤ 0.05 vs. DMEM-incubated impacted controls.
Figure 3.57. Effect of α-MSH, D[TRP]<sub>8</sub>-γ-MSH and dexamethasone on basal production of IL-1β, IL-6 and IL-8.

Bovine articular cartilage explants were treated with DMEM, α-MSH (3.0 µg/ml), D[TRP]<sub>8</sub>-γ-MSH (3.0 µg/ml), or dexamethasone (1.0 µM) for 6 h and cell-free supernatants were collected and analysed for IL-1β (Panel A), IL-6 (Panel B) and IL-8 (Panel C) via commercially available ELISAs (R&D Systems). Results were corrected per gram of tissue tested. Data are presented as Mean ± SEM of n=4 experiments repeated in triplicate. *p<0.05, **p<0.01, ***p<0.001 compared to DMEM-treated controls (Dotted line).
Chapter 4
Discussion
Osteoarthritis is a degenerative joint disorder, characterized by inflammation and cartilage degradation (Pelletier et al., 1991, Shinmei et al., 1991, Lotz et al., 1992, Reboul et al., 1996, Melchiorri et al., 1998, Shlopov et al., 2000, Fernandes et al., 2002, Schuerwegh et al., 2003, Goldring et al., 2008, Rai et al., 2008). It has long been known that the body produces endogenous compounds capable of repressing or resolving inflammation. These include annexin-1 (Flower and Blackwell, 1979, Hannon et al., 2003), galectins (La et al., 2003) and in the context of this thesis, the melanocortin peptides (Star et al., 1995, Ichiyama et al., 1999, Getting et al., 2001, 2002, Scholzen et al., 2003). They have been shown to possess a multitude of actions including inhibition of cytokines, chemokines and NO release from MØ (Star et al., 1995, Getting et al., 1999, 2001 Kalden et al., 1999) and reduction of adhesion molecule expression on the endothelium (Kalden et al., 1999, Scholzen et al., 2003), all these actions help to modulate the host’s inflammatory response. Given the wealth of knowledge generated so far, few studies have looked at the potential anti-inflammatory effect of melanocortin peptides in osteoarthritis.

This thesis investigates the ability of melanocortins to modulate pro-inflammatory cytokines production (such as IL-1β, IL-6, IL-8, MCP-1) and induce anti-inflammatory protein release (HO-1 and IL-10), but also apoptotic pathways induction and cell death from cell line and primary bovine chondrocytes in vitro and in situ. This work has allowed for better understanding of their potential role of melanocortin peptides in modulating some of the inflammatory pathways and cell death aspects of degenerative pathologies such as osteoarthritis, but also to ascertain the role of chondrocytes in this disorder.

Several main conclusions can be drawn from the results presented in this thesis. The first converges on the use of an in vitro cell culture-based model of chondrocyte inflammation that allows studying the effects of inflammatory mediators on chondrocyte metabolism and cytokine and chemokine production. It has been shown that monolayer cultures, yielding high cell density are one of the best tools for studying the cellular and molecular mechanisms of activated chondrocytes (Finger et al., 2004, Goldring, 2004).
Furthermore, this thesis has evaluated whether the human C-20/A4 chondrocytes are a suitable surrogate model for evaluating the effect of anti-inflammatory drugs including melanocortin peptides on inflammatory mediator release from stimulated cells. The human C-20/A4 chondrocytic cell-line, and primary chondrocytes in vitro and in situ (cartilage explants) were investigated for expression of pro-inflammatory cytokines and cartilage degrading MMP production upon activation and the role that these cytokines play in cartilage metabolism (collagen type I and type II production) was also studied. These results suggested a possible role for chondrocytes in cartilage inflammation and damage.

Secondly, functionally active MC receptors were detected in human C-20/A4 chondrocytes and primary bovine articular chondrocytes, which upon activation led to a significant induction of cAMP formation, partial repression of pro-inflammatory cytokine and matrix metalloproteinases production, and marked increase in production of the anti-inflammatory proteins HO-1 and IL-10.

Thirdly, it was concluded that inhibition of MC₃ activity by SHU9119 exacerbates the inflammatory response of the chondrocytes and abolishes the potent anti-inflammatory effects displayed by D[TRP]^8-γ-MSH, therefore suggesting a role for this receptor in modulating some of the inflammatory pathways elicited by activation of C-20/A4 chondrocytes.

Finally, the protective effects of melanocortins were evaluated on TNF-α-induced chondrocyte death and inflammation, as well as identification of their cytoprotective and anti-inflammatory role in mechanically injured cartilage explants. During osteoarthritis ECM matrix degradation leads to tissue hydration that signifies a decrease in extracellular osmolarity and leads to chondrocyte sensitization to external stimuli. An in vitro model (using C-20/A4 chondrocytes) was established mimicking these conditions, which led to the production of pro-inflammatory cytokines and MMPs and subsequently, the anti-inflammatory properties of the melanocortins in this model were confirmed. In all cases a strong correlation occurred with: melanocortin peptide dampening the pro-inflammatory cytokine synthesis/release, inducing anti-inflammatory proteins and cytokines to aid in the resolution of inflammation and significantly diminishing
chondrocyte cell death resulting from both mechanical trauma (in the case of cartilage explants) and the cytotoxic effect of exogenous TNF-α.

Chondrocytes play an important role in maintaining the integrity of cartilage, during osteoarthritis they become activated leading to the release of an array of pro-inflammatory mediators, apoptosis and alterations in the synthesis of ECM (Pelletier et al., 1991, Goldring et al., 1994b, Shlopov et al., 1997, 2000, Goldring et al., 2011). Immortalised chondrocytic cell lines have been developed to serve as easily accessible, reproducible models of chondrocyte function. Given the difficulty in obtaining human tissue, the use of human cell lines can be invaluable, in trying to dissect the inflammatory pathways involved in chondrocyte function and identification of novel treatments for modulating these pathways. Primary cultures of articular chondrocytes isolated from various animal and human sources have served as useful models for studying the mechanisms controlling the response of chondrocytes to various pro-inflammatory and catabolic factors and cytokines (Goldring, 2004). The use of human chondrocytes is problematic and the restricted access to human tissue posses great difficulties for using these cells as a surrogate model. The immortalized chondrocyte cell lines, instead of substituting for primary chondrocytes, may serve as models for increasing knowledge on chondrocyte function not achievable by the use of primary chondrocytes (Finger et al., 2004). Therefore a reproducible source of chondrocytes with human origin would be the most desirable model for studying cartilage function relative to human osteoarthritis (Goldring, 2004) and that is why the human cell line C-20/A4 chondrocytes were used to evaluate chondrocyte’s function and response to inflammatory mediators.
Effect of pro-inflammatory stimuli on nitric oxide release and on chondrocyte death.

Inducible nitric oxide synthase (iNOS) gene expresses a calcium calmodulin-independent enzyme which can catalyse NO production from L-arginine. The induction of iNOS activity has been demonstrated in a wide variety of cell types under stimulation with cytokines and LPS. Recent studies have indicated that human articular chondrocytes express iNOS (Schmidt et al., 2010). C-20/A4 chondrocytes monolayer cultures were stimulated with varying concentrations of \( \mathrm{H}_2\mathrm{O}_2 \), TNF-\( \alpha \), LPS and MSU for 6 h and the ability of these stimuli to activate iNOS to produce NO was tested in conjunction with testing their effect on chondrocyte viability. Substantial amounts of NO, released from C-20/A4 cells following stimulation with \( \mathrm{H}_2\mathrm{O}_2 \) were detected at 6 h post stimulation via the Griess assay, demonstrating that the chondrocytes can react to oxidative stress by activating iNOS (Mendes et al., 2003a,b). In particular, the expression of iNOS has been associated with chondrocytes during the pathogenesis of OA (Grabowski et al., 1997) and the overproduction of nitric oxide was detected in synovial tissue and articular cartilage (Melhiorri et al., 1998). Mendes and colleagues, however demonstrated that \( \mathrm{H}_2\mathrm{O}_2 \) on its own cannot induce iNOS in human articular chondrocyte, but rather mediates IL-1\( \beta \)-induced iNOS expression and activation (Mendes et al., 2003a,b). However, in preliminary studies, \( \mathrm{H}_2\mathrm{O}_2 \) failed to cause significant induction in the release of IL-1\( \beta \). Therefore the ability of C-20/A4 chondrocytes to release nitrate following \( \mathrm{H}_2\mathrm{O}_2 \) activation is not in agreement with previously published work by Mendes et al (Mendes et al., 2003a,b) and could thus be a characteristic of our cell-line.

Inflammation is a characteristic feature of osteoarthritis (Hegemann et al., 2005, Maccoux et al., 2007) and the role of TNF-\( \alpha \), as inflammation-propagator is well documented (Oppenheim et al., 1989; Pelletier et al., 1991; Westacott et al., 1996; Martel-Pelletier et al., 1999). TNF-\( \alpha \) receptors have been detected on the surface of human articular chondrocytes with expression levels significantly increased in osteoarthritic compared to healthy tissue (Westacott et al., 1994). This thesis demonstrates that TNF-\( \alpha \) can activate C-20/A4 chondrocytic iNOS to produce NO in amounts significantly higher than those detected in non-stimulated counterparts. The NO production was maximal following 80.0 pg/ml
but was not significantly different from those observed following stimulation with lower concentrations of the cytokine. This ability of TNF-α to induce nitrite release agrees with findings obtained from TN-α-stimulated canine chondrocytes, where human recombinant TNF-α up-regulates iNOS expression (Rai et al., 2008). It was concluded that TNF-α was able to induce nitric oxide synthesis in chondrocytes, suggesting a possible role of this cytokine in chondrocyte-induced inflammation and possibly matrix metalloprotiensase induction.

To further our understanding of the most appropriate stimulus for activation of C-20/A4 chondrocytes the effect of LPS (the principle component of the outer membrane of Gram-negative bacteria) was determined, as cells have been shown to generally respond to LPS by up-regulating expression of variety of inflammatory cytokines and cytotoxic mediators, such as NO, observed in arthritis (Haglund et al., 2008, Campo et al., 2010). C-20/A4 chondrocyte stimulation by various concentrations of TNF-α and LPS, led to significantly elevated NO production, an event accompanied by a concentration-dependent increase in chondrocyte death. The effect of TNF-α and LPS on this parameter was modest but sufficient for the purposes of this study, thus making them suitable for further evaluation in this study.

Given the role that MSU crystals play in gouty arthritis (McCarty et al., 1966, McGill and Dieppe, 1991, Chilappa et al., 2010) by depositing into the knee joint and driving leukocyte migration and the release of pro-inflammatory cytokines including IL-1β and KC in mouse (Getting et al., 2006), their effect on chondrocytes was investigated. However, MSU crystals were unable to activate these cells at any of the concentrations tested, since a non-significant amount of nitrite release and pro-inflammatory cytokines and chemokines were detected. To date no studies have shown that this cell line can be activated by MSU crystals, although a recent study has shown that MSU crystals can cause an upregulation in COX-2 in primary chondrocytes (Lee et al., 2009). The lack of effect could be due to differences often observed between primary and immortal cell-lines.

However, although primarily thought to be the causative agent for gouty arthritis, there is conflicting evidence over the role they play in OA with a suggestion that urate crystals play an important role in contributing to the onset and/or acceleration of OA (for review see Nowatzky et al., 2010).
Osteoarthritis is accompanied by extended apoptotic chondrocyte death, which has been thought to play a central role in the initiation and progression of the disorder (Blanco et al., 1998; Kim et al., 2000; Aigner et al., 2001). A diverse set of stimuli can trigger the apoptotic process in virtually all eukaryotic cells (Steller, 1995, Thompson, 1995); therefore the cytotoxicity of various concentrations of \( \text{H}_2\text{O}_2 \), TNF-\( \alpha \), LPS and MSU on C-20/A4 chondrocytes via the mitochondrial functionality assay, MTT, which is a convenient assay used to monitor cell death quantitatively rather than qualitatively was investigated. \( \text{H}_2\text{O}_2 \) caused an 80 to 95 \% reduction in cell-viability, even at the lowest concentration used. The reduction in chondrocyte cell-viability due to the endogenous production of excessive amounts of ROS has been previously described (Del Carlo and Loeser, 2002). In addition, \( \text{H}_2\text{O}_2 \) stimulation in C-20/A4 chondrocyte model provides data relevant to chondrocyte death initiated by endogenous ROS production, which has been reported to occur after mechanical trauma (Kurz et al., 2004). The use of this stimulus validated that C-20/A4 chondrocytes were able to respond to oxidative stress; however its use was discontinued for the present study due to the amount of cell death determined. Given the high-level of cell-death attributed to using \( \text{H}_2\text{O}_2 \), the effect of TNF-\( \alpha \) on this parameter was determined. TNF-\( \alpha \) led to a marked concentration-dependent cell death, causing chondrocytes to possibly undergo cell death through recruitment of the mitochondrial pathway of apoptosis thought bcl2 family (Thomas et al., 2000). Its cytotoxicity was highest at 80.0 pg/ml, leading to a near 30 \% reduction in cell viability, whilst lower concentrations, such as 60.0 pg/ml led to a more moderate 15 \% decrease in cell viability and apoptosis. Moreover, the ability of TNF-\( \alpha \) to trigger apoptosis in chondrocytes has been previously confirmed (Li et al., 2011). LPS stimulation caused ~20\% decrease in chondrocyte viability, with lower concentrations (1.0 \( \mu \)g/ml) being more cytotoxic than higher concentrations (10.0 \( \mu \)g/ml). Taken together, these results demonstrate that both TNF-\( \alpha \) and LPS impair mitochondrial function in C-20/A4 chondrocytes.
**C-20/A4 chondrocytes respond to TNF-α and LPS by expressing and releasing pro-inflammatory cytokines.**

Following the identification that C-20/A4 human chondrocytes respond to TNF-α and LPS, the effects of these inflammogens on the release of pro-inflammatory cytokines/chemokines, degradative enzymes and catabolites were evaluated since they are released from chondrocytes upon stimulation (Goldring *et al.*, 2011).

Initially, RT-PCR showed that following stimulation with TNF-α and LPS there was an upregulation in mRNA levels of *IL6* and *IL8* with TNF-α (60.0 pg/ml: a concentration chosen for sub-maximal release of nitrite and cell death) causing a marked increase in both *IL6* and *IL8* expression at all time points evaluated, with a peak at 2 h and decreasing thereafter. Upregulation of pro-inflammatory cytokines has been previously reported in this cell-line (Palmer *et al.*, 2002) and that this observation is translated to primary chondrocytes (Rai *et al.*, 2008). Given that these cells are in monolayers, the fast upregulation of these genes could be explained as they are exposed to the inflammogen in the cell culture media directly, so they can act immediately after stimulation (Rai *et al.*, 2008).

After identifying the 6 h time-point as suitable for subsequent experiments, since it caused a sub-maximal up-regulation in IL-6 and IL-8 mRNA, different concentrations of TNF-α and LPS were evaluated. TNF-α caused a bell-shaped response with 60.0 pg/ml causing a sub-maximal upregulation in the cytokines genes. These results are in accordance with published data, where TNF-α has been shown to stimulate the production of variety of pro-inflammatory cytokines (Shinmei *et al.*, 1991; Shlopov *et al.*, 2000; Rai *et al.*, 2008).

Even though LPS has been used to induce cartilage degradation for the last 30 years (Jasin, 1983, Morales *et al.*, 1984, Tian *et al.*, 1989), the expression of its membrane receptor (TLR4) on chondrocytes and their response to LPS have only been recently investigated (Kim *et al.*, 2006; Haglund *et al.*, 2008). In order to gain a further insight into how chondrocytes respond to this pro-inflammatory stimulus, C-20/A4 chondrocytes were stimulated with increasing concentrations of LPS (0.1 – 1.0 µg/ml) and detected a concentration-dependent elevation in *IL-6* and *IL-8* expression, peaking at 1.0 µg/ml, which was in accordance with other studies (Bobacz *et al.*, 2007).
Following the identification of an upregulation in IL-6 and IL-8 mRNA, an ELISA assay was used to ascertain whether this message was genuinely translated into protein. IL-6 and IL-8 release was detected at 2, 6 and 24 h after stimulation with TNF-α and LPS and is in agreement with previous studies (Campo et al., 2008, Henrotin et al., 1996, Lotz et al., 1992). For this part of the study, the concentration range of LPS was broadened by including 3.0 µg/ml and 10 µg/ml, in order to further investigate the effect of the concentration LPS on the production of these cytokines and to identify if a bell-shaped response could be observed. TNF-α and LPS caused a time and concentration-dependent effect with significant elevations in both IL-6 and IL-8 production. However, at 24 h post stimulation, all concentrations of LPS were equipotent, thus suggesting that a plateau might be reached at that time point. Previous studies have demonstrated that production of IL-1β, IL-6, IL-8, and TNF-α was responsive to a wide range of LPS concentrations (0.1 ng/ml – 10.0 µg/ml) and that these cytokines were first detected at 1 – 4 h and reached a plateau levels after 6 h (DeForge et al., 1992).

The importance of these findings is related to the fact that the C-20/A4 cell-line responds to inflammatory stimuli in a similar fashion to primary cells/cartilage (Henrotin et al., 1996; Campo et al., 2008). Taken together, these findings demonstrated that the C-20/A4 chondrocytes are capable of expressing and producing IL-6, therefore suggesting that IL-6 may also play a physiological role in cartilage. An interesting finding of this study is the novel expression of IL-8 by the human chondrocytic cell line, showing significant up-regulation following TNF-α-stimulation. Currently, the role of this chemokine has not been thoroughly investigated in human cartilage – a weak IL-8 mRNA signal had been detected in normal untreated chondrocytes (Lotz, 1992; David, 2007) and freshly isolated chondrocytes have been shown to spontaneously release detectable amount of IL-8 (Fan et al., 2005). Our data confirm these findings suggesting that IL-8 and other CXC chemokines, may trigger the release of matrix degradative enzymes and therefore the subsequent cartilage destruction (Lotz et al., 1992).

In order to broaden our investigative spectrum of pro-inflammatory cytokines, we looked at IL-1β and MCP-1 mRNA and protein in response to TNF-α and LPS stimulation. These cytokines were chosen since IL-1β is active locally within cartilage and has been shown to play an important part in OA initiation and
progression (Fernandes, 1999; Kobayashi, 2005; Lopez-Armada, 2006; Kapoor, 2011). Moreover, IL-1β has been immunolocalized in chondrocytes taken from human OA cartilage with histologically confirmed degenerative changes (Dayer, 2002; Goldring et al., 2011) with its role as propagator of inflammation leading to cartilage degradation being well documented (Westacott, 1996; Martel-Pelletier, 1999; Attur, 2000; Moldovan, 2000; Lopez-Armada, 2006; Kapoor, 2011).

MCP-1 levels were then determined following activation of C-20/A4 chondrocytes with varying concentrations of TNF-α and LPS. Although its main role is monocyte chemotaxis and activation (Zachariae et al., 1990), it is involved in a vital pathologic relationship between chondrocytes and the synovium, where through the release of MCP-1, chondrocytes “invite” mononuclear phagocytes into the cartilage, resulting in rheumatoid pannus formation onto the articular surface (Villiger et al., 1992). Here, TNF-α and LPS caused MCP-1 to be released with low levels of detection at early time-points and detectable amounts at 6 h. The time-frame for MCP-1 release observed in chondrocytes is supported by previous studies, which demonstrated that maximal production of MCP-1 was detected 4 h post induction of zymosan-peritonitis (Ajuebor et al., 1998). In addition, work by Lotz and colleagues, revealed using in situ hybridization of cartilage organ cultures that chondrocytes in the superficial tangential zone responded within 2 h of stimulation with IL-1 by significantly increasing MCP-1 transcripts and chondrocytes in deeper layers responded by 4 h and reached maximum MCP-1 expression by 8 – 12 h (Villiger et al., 1992). This release of MCP-1 from chondrocytes highlights the fact that monocytes can migrate into the joint, therefore promoting cartilage degradation in the later stages of OA. Therefore, targeting these cytokines may play an important role in stopping the development of this pathology.

Following the identification of cytokine release from activated chondrocytes their effect on cartilage degradation processes was determined (Brinckerhoff, 1992, Goldring et al., 2008, 2011, Grassel et al., 2009), consequential from the up-regulation and activation of MMPs (Shlopov, 1997, 1999, 2000). The effect of TNF-α and LPS on matrix degradative enzymes was investigated as these collagenases are unique enzymes capable of cleaving interstitial collagen
(Gadher et al., 1990, Kevorkian et al., 2004). Generally, collagenase expression is believed to be NF-κB dependent, a pathway that is also employed by the TLR receptors (Zhang et al., 2008). TNF-α and LPS caused a concentration-dependent bell-shaped increase in MMP1 and MMP13 mRNA levels in C-20/A4 chondrocytes. These results are in agreement with a previous study, in which a four-fold increase in MMP1 was observed following stimulation with IL-1β (Grange et al., 2006) although it is reported for the first time here that MMP13 is also upregulated in these cells. This upregulation in MMP1 and MMP13 has previously been shown in primary chondrocytes (Shlopov et al., 2000; Rai et al., 2008). This was substantiated by the observation, that the levels of these collagenases increased with extended incubation times observed here and previously (Rai et al., 2008). Interestingly, MMP1 expression was significantly up-regulated in the stimulated samples, showing high similarity to the expression profile of MMP1 in normal and OA human knee cartilage, and demonstrating the opposite of what takes place in the human hip during end stages of OA, where MMP1 is down-regulated (Kevorkian et al., 2004). However, the current increase in the expression could suggest a possible role of MMP1 in this in vitro C-20/A4 chondrocyte model, and suggest that it resembles more the processes that occur in knee OA, rather than that of the hip OA.

Statistically-significant changes were observed in the expression profile of MMP3 with decreased expression, in a concentration-dependent manner, following a stimulation with both TNF-α and LPS for 6 h. This reduction in MMP3 has not been previously reported in this cell-line and correlates with data in knee OA cartilage (Bau et al., 2002). The down-regulation of MMP3 in this in vitro model is interesting especially when compared to the reported increase in the MMP3 levels in the synovial fluid of RA patients (Matsuno et al., 2001). However, MMP3 is reported to be the most highly expressed matrix metalloproteinase in normal knee cartilage, suggesting a maintenance function of this enzyme in normal cartilage metabolism, which was dysregulated in OA (Bau et al., 2002; Kevorkian et al., 2004).

Further examination of the collagenases showed that the main catabolic enzyme for type II collagen found in cartilage, MMP13, was increased in TNF-α-stimulated C-20/A4 chondrocytes, which is consistent with the current belief that
it is the principal collagenase in OA (Kevorkian et al., 2004). Previous studies have demonstrated that IL-6 promotes cartilage degradation (Shlopov et al., 2000) by directly inducing MMP1 and MMP13. Here, it’s demonstrated that there is simultaneous up-regulation of IL-6 and MMP13, therefore confirming the hypothesis that IL-6 and MMP13 are involved in the progression of OA. Overexpression of these genes in the cartilage may further induce an increase of hypertrophic chondrocytes (Goldring et al., 2011, Tchetina et al., 2005) resulting in the destruction of the upper-layer cartilage matrix and progression of cartilage degeneration.

In healthy articular cartilage, chondrocytes are actively maintaining the expression and ratio of collagens and proteoglycans (Hall, 1998). Chondrocytes are very sensitive to pro-inflammatory cytokines, an observation supported by this study. Studies have reported that pro-inflammatory cytokines either reduce or enhance the production of type II collagen, a marker of normal function of chondrocytes (Ho et al., 2006). Therefore to determine if the cell-line responded in a similar fashion to primary cells, the direct effect of TNF-α on the expression of the cartilage specific collagens was determined by RT-PCR. TNF-α stimulation caused COL1A1 and COL2A1 levels to decline significantly, therefore inhibiting the chondrocyte compensatory synthesis pathways, required to restore integrity of the degraded matrix (Goldring and Goldring, 2004). It is important to point out, that TNF-α did not influence the differentiation indicator ratio of COL2A1:COL1A1 detected in unstimulated C-20/A4 cells, which is an important observation as dedifferentiated cells would be undesirable for the purposes of this study.

To ensure that our in vitro cell-line system responded in a similar fashion to primary cells the effect of the glucocorticoid dexamethasone and the NSAID Indomethacin was evaluated given the role they play in the treatment of inflammatory pathologies. Glucocorticoids are powerful anti-inflammatory molecules shown to be able to repress transcriptional activation of genes including IL1, IL-6, IL-8, TNF-α, γ-interferon, colony stimulating factor (CSF)-1/macrophage, granulocyte macrophage (GM)-CSF (Taniguchi, 1988). Most of these genes are activated by the transcription factors NF-κB and AP-1, and their down-regulation confirms that glucocorticoids are interfering with these pathways
NSAIDs exert their effects by inhibiting COX enzyme (part of the arachidonic acid cascade) and reducing prostaglandins leading to a diminished inflammation and pain (Vane, 1971, 1976). At present only COX-1 and COX-2 are clinically relevant, with COX-1 regarded as housekeeping enzyme responsible (via prostaglandins and thromboxane A2) for physiological functions including protection of gut mucosal integrity and vascular homeostasis (Chen et al., 2008), whilst COX-2 appears to be a more important mediator of inflammation and thus a key factor in arthritic pain (Chen et al., 2008).

Dexamethasone and Indomethacin were administered 30 min prior to TNF-α stimulation of C-20/A4 chondrocytes. Dexamethasone and indomethacin have previously been shown to inhibit cytokine production in other cell systems (Mukaida et al., 1991). Glucocorticoids activate intracellular receptors that then bind to glucocorticoid-responsive elements in the promoters of various genes, or inhibit NF-κB translocation in the nucleus (Vayssiere et al., 1997). Furthermore, they inhibit AP-1 DNA binding ability and therefore block the respective gene expression (Vayssiere et al., 1997). In this study they were shown to inhibit TNF-α (60.0 pg/ml) induced expression of IL-6 and IL-8 mRNA and protein, an effect accompanied by a abrogation of the expression of MMP1, MMP3 and MMP13 over the time-course. This ability to modulate inflammatory pathways in this cell-type was in agreement with previous studies utilizing primary chondrocytes (Richardson and Dodge, 2003). Both the glucocorticoid dexamethasone and the NSAID indomethacin completely abrogated the production of IL-6 and IL-8 at all time points tested, and in doing so they negatively surpassed even the basal levels of production of these cytokines detected in unstimulated cells. However, although this effect may be desireable in management of acute inflammation, in chronic inflammatory diseases such as OA, this could lead to supression of the HPA axis and lead to impaired wound healing, Cushings syndrome and opportunistic infections (Gupta et al., 2000; Alekseev et al., 2001; Dorscheid et al., 2006).

Data generated here looking at exempler cytokines, shows that TNF-α and LPS alone trigger a cascade of cytokines in this in vitro model. The results obtained from the ELISA and the PCR revealed a minimum 2-fold increase in inflammatory marker genes in these activated C-20/A4 chondrocytes. Of importance was the
observation that classical anti-inflammatory drugs (dexamethasone and indomethacin) were able to modulate these pathways. These data suggests that its possible to use this cell-line as a surrogate in vitro model for investigating inflammatory pathways within these cells and to evaluate the effects of the melanocortin peptides.

**Melanocortin receptor expression in C-20/A4 chondrocyte and the role of melanocortins in modulation of pro-inflammatory cytokine production**

Melanocortin peptides have potent antipyretic and anti-inflammatory effects (Grabbe, 1996; Getting et al., 1999, 2001, 2008, 2009; Getting 2002, 2006, Luger, 2000), which they deliver via activation of a family of 7TM-GPCRs (Catania et al., 2004). These are termed melanocortin receptors and to date five subtypes have been identified (Getting et al., 2009). Previous research within the field of inflammation has highlighted that these peptides can modulate the effect of several pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6, and IL-8 (Catania et al., 1999; Grassel et al., 2009) and they are inducers of the anti-inflammatory cytokine IL-10 (Redondo et al., 1998; Lam et al., 2005). This study tested the hypothesis that targeting melanocortin receptors may provide a novel therapeutic approach to treatment of chondrocyte inflammation, such as that observed in OA.

For this purpose, pharmacological and molecular techniques were used employing the melanocortin receptor pan-agonist α-MSH (Catania et al., 2006; Getting, 2002, 2006; Getting et al., 2009; Rajora et al., 1996, 1997) and the selective MC3 agonist D[TRP]8-γ-MSH (Grieco, 2000; Getting, 2006; Getting et al., 2008, 2009). To date, two receptors have been identified to mediate the anti-inflammatory effects of melanocortin peptides, namely the MC1 and MC3 (Getting, 2002, 2006, Getting et al., 2009). However, some evidence points towards a role played by MC5 in inflammation, since its expression has been detected on B-lymphocytes (Buggy, 1998) and T-lymphocytes (Taylor and Namba, 2001), suggesting a potential role in immuno-modulation.

Initially, expression of MC1, MC3 and MC5 was determined in C-20/A4 chondrocytes, reported here for the first time with a strong signal MC1 and MC3, and a very faint signal corresponding to MC5. Recently, gene expression of MC1
was identified in the human chondrosarcoma cell line HTB-94 (Yoon et al., 2008), and MC1, MC3 and MC5 transcripts have been detected in primary articular chondrocytes (Grässel et al., 2009). Whilst the detection of MC1 is in agreement with previous studies, to our knowledge the detection of MC3 expression in human chondrocytes is novel. Given the apparent disparity in the results observed and those generated previously in primary cells, western blotting was used to determine if mRNA for MC1 and MC3 was translated into protein.

Following the identification of mRNA and protein for MC1,3 and mRNA for MC5, the functionality of the receptors was determined by evaluating a panel of melanocortin peptides (displaying different receptor selectivity) on cAMP accumulation as detected by EIA. Melanocortin receptors are positively coupled to adenylate cyclase, which upon activation causes increase in intracellular cAMP formation (Catania et al., 2006; Gantz et al., 2003; Getting et al., 2009). In order to determine the functionality of the melanocortin receptors, at first we tested whether the MC1 receptor agonist α-MSH, the MC3 selective agonist D[TRP]$^8$-γ-MSH, and the MC5 selective agonists SHU9119, PG901 and PG911 could induce cAMP accumulation in the human C-20/A4 cells. Our functional studies showed that both α-MSH and D[TRP]$^8$-γ-MSH were able to elicit a significant and concentration-dependent increase in cAMP formation, this effect being observed in C-20/A4 chondrocytes for the first time. Whilst α-MSH has previously been shown to induce cAMP accumulation in chondrocytes (Grassel et al., 2009) the ability of the MC3 agonist D[TRP]$^8$-γ-MSH to induce increases in cAMP has not been previously demonstrated in chondrocytes. This increase in cAMP occurred in a bell-shaped manner and is in agreement with previous studies utilizing these peptides in other cell-types (Getting et al., 2006).

Given that MC5 mRNA was observed in C-20/A4 chondrocytes and has previously been shown to be expressed on primary articular chondrocytes (Grassel et al., 2009), the effect of selective MC5 agonists PG901, PG911 (Grieco et al., 2002) and the mixed agonist/antagonist SHU9119 (Hruby et al., 1995) was evaluated. Treatment of cells with these peptides at all the concentrations evaluated did not cause an increase in cAMP accumulation. This lack of effect of the selective MC5 agonists PG901 and PG911, was perhaps due
to the weak mRNA expression of this receptor, not translated to protein. Whilst we cannot completely rule out the expression of a functionally active MC5 in the C-20/A4 cell model, given the fact that more selective compounds maybe developed in the future, which would allow further investigation of this receptor. At present, it is highly unlikely that this receptor plays a role in modulating the effects of the melanocortin peptides given the low expression level and lack of functionality displayed here in this model.

Given that α-MSH and D[TRP]8-γ-MSH significantly elevated cAMP levels, the peptides were evaluated in the presence of the MC3/4 antagonist SHU9119 (Fan et al., 1997) used at a concentration previously shown to inhibit the cAMP accumulation elicited by these peptides (Getting et al., 2006), thus allowing identification of whether MC1 or MC3 was involved. Incubation of the C-20/A4 chondrocytes with SHU9119 was able to inhibit D[TRP]8-γ-MSH confirming previous findings in other cell types (Getting et al., 2006) and in models of inflammation (Getting et al., 2008, Leoni et al., 2008, Patel et al., 2010), whereby D[TRP]8-γ-MSH mediates its effects via MC3. Not surprisingly, SHU9119 (10.0 µg/ml) failed to block α-MSH at all concentrations, except 3.0 µg/ml, which caused an extremely modest reduction in cAMP levels. At MC3, the peptides ACTH1-39, α-MSH, β-MSH and γ-MSH are equipotent (Getting, 2006) and even though α-MSH preferentially activates MC1, it cannot be excluded that, at this concentration, some of the increase in cAMP observed with α-MSH may be in part due to activation of the MC3.

Numerous studies have highlighted the ability of melanocortin peptides to inhibit cytokine release in vitro (Lam et al., 2005, 2006) and also in vivo models of inflammation (Ceriani et al., 1994, Getting, 2002, Getting et al., 2003, 2006, 2008). However, to date only one study has investigated the effect of melanocortin peptides on chondrocytes (Grässel et al., 2009) and none using the selective MC3 agonist D[TRP]8-γ-MSH. C-20/A4 chondrocytes were treated with α-MSH (3.0 µg/ml), D[TRP]8-γ-MSH (3.0 µg/ml), and SHU9119 (10.0 µg/ml) to ascertain their effect on basal release of cytokines from these cells. In order to determine the effect of the petides in the absence of detectible inflammation, IL-
1β, IL-6, IL-8 and MCP-1 levels were determined by ELISA and showed that none of the peptides caused an elevation in basal cytokine release from these cells.

The effect of melanocortin peptides on TNF-α induced inflammatory markers, was then evaluated since it has been shown to be involved in activation of chondrocytes leading to the degradation of cartilage within the knee joint. α-MSH has long been known to suppress inflammation by down-regulating the expression of pro-inflammatory cytokines, and to have anti-inflammatory and immuno-modulatory actions in rodent models of inflammation in a corticosterone-independent manner (Getting et al., 1999). Here it inhibited TNF-α-induced release of IL-1β, IL-6, IL-8 and MCP-1 from C-20/A4 chondrocytes in a concentration-dependent manner. The peptide displayed potent anti-cytokine effects at both 2, and 6 h, a similar observation that was noted in primary murine peritoneal macrophages (Getting et al., 1999) and macrophage cell-lines (Lam et al., 2006). The peptide did not elicit any effect following 24 h incubation. The suppression of IL-1β, IL-6 and IL-8 by α-MSH in C-20/A4 chondrocytes is in accordance with the overall anti-inflammatory and protective capacity of the peptide (Catania et al., 2004).

Given the elevation in cAMP observed with the MC3 agonist D[TRP8]-γ-MSH, it was evaluated in this model. Here the effect of D[TRP8]-γ-MSH (0.1 – 30.0 µg/ml) on modulation of pro-inflammatory cytokine release from human C-20/A4 chondrocytes activated by TNF-α was determined. C-20/A4 chondrocytes were treated with the selective melanocortin peptide for 30 min prior to stimulation with TNF-α, and subsequently incubated for 2-24 h, when supernatants were collected and analysed for IL-1β, IL-6 and IL-8 release. D[TRP8]-γ-MSH showed a bell-shaped inhibition of IL-1β release with 3.0 µg/ml and 10.0 µg/ml being consistently the most potent concentrations, causing ~ 70-80 % inhibition. Similarly, a bell-shaped response in IL-6 was observed following 2 and 6 h of incubation, peaking at 3.0 µg/ml D[TRP8]-γ-MSH, however, when the treatment was continued for 24 h, the peptide showed a concentration dependent inhibition of IL-6. IL-8 production was inhibited by D[TRP8]-γ-MSH in a concentration-dependent manner at all time points, with 10.0 and 30.0 µg/ml being the most potent with a 70-80% reduction in this chemokine following TNF-α stimulation.
These data highlight for the first time the ability of this peptide to inhibit cytokine release from chondrocytes. The peptide displayed efficacy at all time-points evaluated and was still active at 24 h post treatment, correlating with the findings of this peptide in urate crystal-induced inflammatory cytokine release from macrophages (Lam et al., 2005) The maximal anti-inflammatory effect at 2 and 6 h post stimulation was reached by 3 and 10.0 μg/ml of D[TRP]8-γ-MSH concentrations previously shown to be effective (Getting et al., 2006, 2008) with a 70-80 % inhibition of IL-1β release.

This ability of the peptide to suppress cytokine release was recently confirmed in a model of LPS-induced lung inflammation (Getting et al., 2008). With respect to models of arthritis α-MSH has been shown to repress experimental adjuvant-induced arthritis in rats (Ceriani et al., 1994), whilst a recent study by the Perretti group has highlighted the importance of the MC3 agonist D[TRP]8-γ-MSH in a model of serum transfer arthritis where the peptide was effective in wild type mice but not in MC3-/- null mice (Patel et al., 2010). However, neither of the selective MC5 agonists (MC3/4 antagonists) PG901 and PG911 was able to cause significant decrease of TNF-α-induced pro-inflammatory cytokines, which together with the inability of the peptides to elicit cAMP increases, suggesting that this receptor is not functionally active in C-20/A4 chondrocytes.

The anti-inflammatory effects of α-MSH and D[TRP]8-γ-MSH were evaluated on IL-6 and IL-8 transcription levels in the presence or absence of the MC3/4 antagonist SHU9119, at a dose previously shown to abrogate the inhibitory effects of γ2-MSH on chemokine release (10.0 μg/ml) (Getting and Perretti, 2000). α-MSH has been shown previously shown to suppress an array of inflammatory cytokines including TNF-α (Rajora, 1997 ; Delgado Hernandez, 1999) and IL-1β (Getting et al., 2003). α-MSH has tremendous effect on chemotaxis, further supported by the finding of Luger’s group that this melanocortin peptide inhibits the production and release of IL-8 (Brzoska et al., 1999). Our study demonstrates and confirms the anti-inflammatory effect of not only α-MSH, but also D[TRP]8-γ-MSH in human C-20/A4 chondrocytes. The pre-treatment of C-20/A4 chondrocytes with α-MSH (3.0 μg/ml) prior to TNF-α stimulation caused an ~30 % inhibition in the expression of IL-6 (35 %) and IL-8
(25 %), therefore supporting the collected evidence that this peptide and its putative receptor have marked impact on IL-6 and IL-8 regulation. Additionally, α-MSH not only inhibited the transcription of these genes, but also the protein release of IL-6, IL-8 and MCP-1 from the cells. Cytokine ELISAs showed that there was a 70 %, 60 % and 21 % reduction in IL-6, IL-8 and MCP-1 protein levels released from C-20/A4 chondrocytes. The ability of α-MSH to exert anti-inflammatory actions has been well documented (Martin, 1991; Lipton, 1997, 1999; Catania, 1999), whereas the use of the mixed MC3/4 antagonist SHU9119 (Hruby et al., 1995; Fan et al., 1997; Getting et al., 2006) did not affect the observed anti-cytokine effects of α-MSH, suggesting that the latter must be preferentially activating MC1 in order to exert its effect.

D[TRP]8-γ-MSH was also able to diminish pro-inflammatory cytokines expression causing 27% and 36% inhibition of TNF-α-stimulated IL-6 and IL-8 transcription, respectively. Furthermore, similarly to the action of α-MSH, D[TRP]8-γ-MSH led to 68 %, 45 % and 26 % reduction in IL-6, IL-8 and MCP-1 production, respectively, an observation in accordance with other studies exemplifying the anti-inflammatory and anti-migratory effects of D[TRP]8-γ-MSH on cultured Mø both in vitro and in vivo (Getting et al., 2006). An important finding was that SHU9119 completely obliterated the effect of D[TRP]8-γ-MSH not only on cytokine transcription levels, but also on the synthesis and release of IL-6, IL-8 and MCP-1 from C-20/A4 cells compared to the effect of D[TRP]8-γ-MSH alone. These data, together with the functional studies of MC1 and MC3 receptor activation, highlight not only the anti-inflammatory properties of α-MSH and D[TRP]8-γ-MSH, but also confirms that both MC1 and MC3 might be the main targets for inflammation modulation in C-20/A4 chondrocytic system. Of interest is the fact that unlike dexamethasone and indomethacin, neither of the melanocortin peptides caused a complete abrogation of pro-inflammatory cytokine production. In contrast, they modulated the production of the tested cytokines, therefore allowing for some level of synthesis from the chondrocytes.

A novel finding of this study is the expression of functionally active MC3 on C-20/A4 chondrocytes and that agonism of this receptor modulates the inflammatory response of TNF-α-activated human C-20/A4 chondrocytes.
However in this cell-line no one has ever investigated the effect of melanocortin peptides or their receptors on matrix metalloproteinases expression following TNF-α stimulation. We have shown that TNF-α potently up-regulates the expression of MMP1 and 13 and that there is an upregulation in pro-inflammatory cytokines confirming previous findings (Fernandes et al., 2002; Martel-Pelletier et al., 1999). α-MSH pre-treatment of TNF-α-activated C-20/A4 chondrocytes led to a marked 48 % reduction in transcription of MMP1, one of the interstitial collagenases, significantly up-regulated in human osteoarthritic cartilage compared to healthy tissue (Reboul et al., 1996; Kevorkian et al., 2004).

Collagenase 3, or MMP13, is highly up-regulated in chondrocytes isolated from human osteoarthritic chondrocytes (Shlopov et al., 1997) and following TNF-α stimulation (Rai et al., 2008), and was significantly down regulated by α-MSH (3.0 µg/ml) in our model of TNF-α-activated C-20/A4 chondrocytes. The melanocortin peptide down-regulated the expression of MMP13 by 67 %, compared to TNF-α-stimulated levels. These findings are in agreement with a recent study showing that α-MSH can inhibit TNFα-induced MMP13 expression in the chondrosarcoma cell line HTB-94 (Yoon et al., 2008).

In order to additionally confirm the involvement of MC1 and MC3 in the transmission of these effects, SHU9119 was added in conjunction with α-MSH, but no significant effect was observed on the expression of either MMP1 or MMP3. However, an interesting observation was made when analysing the effect of this combination on MMP13 expression. RT-PCR showed that SHU9119 and α-MSH synergistically inhibited the expression of this collagenase, leading to 87 % reduction compared to TNF-α-stimulated levels, thereby suggesting other mechanism by which this combination might affect the expression of this particular collagenase.

Similar results were obtained following pre-treatment of chondrocytes with the selective MC3 agonist. D[TRP]8-γ-MSH was more potent than α-MSH in reducing MMPs expression, with reductions of 89 %, 76 % and 92 % in MMP1, MMP3 and MMP13 expression, respectively, compared to levels detected following TNF-α treatment. However, when cells were treated with SHU9119 and D[TRP]8-γ-MSH,
the effect was completely attenuated in all cases, confirming the involvement of MC₃ in the modulation of degradative matrix metalloproteinases. All these actions contribute to a local attenuation of the host’s inflammatory response. Given the wealth of knowledge generated so far, few studies have looked at the potential of the melanocortins in inducing anti-inflammatory mediators in chondrocytes (Iannone et al., 2001; Fernandes et al., 2002).

In contrast to the suppressive effects of endogenous melanocortin peptides on pro-inflammatory cytokines production and release, they have been shown to elicit significant elevations in the production of the anti-inflammatory cytokine with potent immuno-suppressive properties, IL-10 (Bhardwaj et al., 1996; Redondo et al., 1998; Lam et al., 2006). The importance of IL-10 in melanocortin receptor biology was first demonstrated in a model of contact hypersensitivity, where an antibody against this cytokine abrogated the protective action attained by α-MSH application (Grabbe et al., 1996). The anti-inflammatory effect of α-MSH observed here and in other models, could be dependent on IL-10 induction, given that α-MSH was inactive in IL-10 knock out mice in a murine model of allergic airway inflammation (Raap et al., 2003). This study sought to reveal, whether α-MSH and D[TRP]⁸-γ-MSH could stimulate the production of IL-10 in C-20/A4 chondrocytes in the presence and absence of TNF-α. Additionally, the ability of SHU9119, PG901 and PG911 was tested in order to investigate whether these peptides could stimulate their cognate receptor (MC₅) to induce IL-10 synthesis. Human chondrocytes from healthy and osteoarthritic cartilage have been shown to express the anti-inflammatory cytokine IL-10 and its putative receptor IL10R (Iannone et al., 2001), which upon interacting down-regulate TNF-α-induced MMP1 and MMP13 (Shlopop et al., 2000). Here, we have demonstrated, that treatment with α-MSH and D[TRP]⁸-γ-MSH (3.0 µg/ml), but not SHU9119 (10.0 µg/ml), PG901 and PG911 (3.0 pg/ml) leads to significant increase in basal IL-10 release from C-20/A4 chondrocytes. In addition, it was apparent that the response of the chondrocytes was time-dependent, with both α-MSH and D[TRP]⁸-γ-MSH causing maximal induction of IL-10 at 6 h post-stimulation. α-MSH was significantly more potent than D[TRP]⁸-γ-MSH at inducing IL-10 although both peptides elevated basal levels of IL-10 as early as 2 h post-administration, and maintained them for 24 h. These results demonstrate that in
part melanocortin peptides can exert a homeostatic control over chondrocyte physiology with the ability to induce anti-inflammatory cytokines. This therefore suggests a possible role in modulating basal levels of pro-inflammatory synthesis in this cell type even when no apparent inflammation is occurring.

Given the induction of basal IL-10 by the melanocortin peptides, their effect were investigated over a concentration range in the presence of TNF-α-induced chondrocyte inflammation over a time-course. α-MSH caused a concentration-dependent bell shaped response with maximal release of IL-10 caused by 1.0 µg/ml at early time-points and 10.0 µg/ml at later time points (24 h), whilst D[TRP]8-γ-MSH (30.0 µg/ml) caused a maximal release of IL-10 at 2 h post-stimulation. The ability of α-MSH and D[TRP]8-γ-MSH to trigger the production of IL-10 clearly suggests that activated melanocortin receptors may have crucial anti-inflammatory properties, conducted through activation of this cytokine.

The anti-inflammatory protein HO-1 has been implicated in the protection against tissue injury and is modulated by cytokines such as TNF-α (Fernandes et al., 2003). It has been shown to be expressed and functionally active in human osteoarthritic chondrocytes from OA tissue with IL-10 shown to modulate its production (Lee and Chau, 2002; Fernandes et al., 2003). A potential link between melanocortin receptor-dependent cAMP formation and HO-1 induction has previously been identified (Lam et al., 2005). This idea stemmed from the fact that cAMP analogues have been shown to induce HO-1 in rat hepatocyte culture (Immenschuh et al., 1998). To address this, the human C-20/A4 chondrocytes were employed to monitor alteration in HO-1 protein production, following incubation with α-MSH and D[TRP]8-γ-MSH at 3.0 µg/ml in the presence and absence of TNF-α. C-20/A4 chondrocytes produced detectable basal levels of HO-1, and melanocortin peptides were able to provoke a marked up-regulation of HO-1 evident at 6 h post-incubation with the melanocortin receptor pan-agonist α-MSH and the synthetic MC3/4 agonist D[TRP]8-γ-MSH. Interestingly, when chondrocytes were pre-treated with the melanocortin peptides prior to stimulation with TNF-α, there was notable elevation in HO-1 production. Together with the fact that TNF-α moderately, but significantly induced the production of HO-1, these results are in accordance with published data.
(Wagener *et al*., 2003). The downstream sequence of events currently remains unclear, and further investigation would be needed to elucidate the action of melanocortin receptor signalling in chondrocytes.

**Chondrocyte apoptosis and the protective effect of melanocortin peptides**

Chondrocytes are the only cell type present within articular cartilage and thus chondrocyte apoptosis plays an important part during the processes of cartilage development, aging and in cartilage pathologies (Blanco *et al*., 1998). Chondrocytes have been shown to be susceptible to endogenous degradative stimuli, such as TNF-α and IL-1β by up-regulating the synthesis of pro-inflammatory cytokines and matrix metalloproteinases, inhibiting collagen and proteoglycan synthesis, therefore causing loss of cartilage (Ismail *et al*., 1992; Martel-Pelletier *et al*., 1999; Fernandes *et al*., 2002; Kapoor *et al*., 2011). The importance of apoptosis has been identified with an increase in the number of apoptotic chondrocytes in osteoarthritic lesional than in non-lesional cartilage (Kim *et al*., 1999; Kim *et al*., 2000; Kouri *et al*., 2000; Hashimoto *et al*., 1998; Kirsch *et al*., 2000). In addition, chondrocyte apoptosis and the reduction of tissue cellularity represent an important step in the pathogenesis of cartilage degradation (Blanco *et al*., 1998; Maneiro *et al*., 2003).

Currently, it remains unclear which pathways induce apoptosis and are responsible for the loss of chondrocytes and subsequent cartilage degradation. DeWolf and colleagues observed that TNF-α (30.0 ng/ml) stimulated caspase-3 driven apoptosis in human chondrocytic cell line (Nuttal *et al*., 2000) and that TNF-α induced apoptosis of bovine chondrocytes *in vitro* (Schuerwegh *et al*., 2003). In our *in vitro* study, we demonstrate that TNF-α potently induces chondrocyte apoptosis, suggesting that this is part of the mechanism of cartilage destruction, thereby substantiating those existing data. TNF-α concentrations (60.0 – 80.0 pg/ml) caused approximately 28% of the C-20/A4 chondrocytes to die, whereas lower concentrations caused around 10-15 % rate. Clinical studies have demonstrated that pathophysiological concentrations of TNF-α detected in OA synovium of patients with severe disease progression are in the range of 1.0 – 10.0 ng/ml (Westacott *et al*., 1990), much higher than the concentrations used
in this study. One reason for using lower concentrations is that chondrocytes in vivo die in the context of extracellular matrix, which may physically limit the levels of pro-inflammatory cytokines reaching the chondrocytes as opposed to in vitro experiments, where the chondrocytes are cultured in monolayer, allowing for fast and equal distribution of TNF-α to all chondrocytes, thereby increasing susceptibility of the chondrocyte to undergo apoptosis in response to TNF-α.

Moreover, the expression of p55 TNF-α receptor has also been localized in areas of osteoarthritic lesions of human cartilage (Webb et al., 1997), and given that the pro-inflammatory cytokine TNF-α is particularly important in the pathophysiology of cartilage disease, I aimed to further investigate the precise role it plays in chondrocyte apoptosis. It was confirmed that TNF-α modulates the activation of apoptotic pathways in human C-20/A4 chondrocytes and may be partially dependent on the activation of caspase-3 and -7. Western blot analysis showed that there was a significant 24 % increase in the protein levels of the activated executioner caspase-3 (Asp-175; 17, 19 kDa) following treatment of the C-20/A4 chondrocytes with TNF-α for 6 h. In addition, TNF-α treatment (60.0 pg/ml; 6 h) led to marked 5.7-fold increase in caspase-3/7 activities, which was confirmed by Caspase-Glo® 3/7 assay analysis. These results are an important finding and are not in accordance with the work by the Blanco group, who detected increased mRNA and protein levels of both caspase-3 and -7 in cultured human OA chondrocytes stimulated with TNF-α, but protein analysis detected only the intermediate, inactive forms of these enzymes (Lopez-Armada et al., 2006). Contrary to those reports, recent studies have demonstrated that TNF-α causes enhanced chondrocyte apoptosis by increasing capsase-3/7 activities (Nuttal et al., 2000; John et al., 2007; Kayal et al., 2010).

Role of melanocortins in prevention of pro-inflammatory cytokine-induced apoptosis.

Following the identification of α-MSH’s and D[TRP]8-γ-MSH’s ability to markedly reduce the synthesis and production of pro-inflammatory cytokines, as well as down-regulating degradative matrix metalloproteinases expression, their effect on TNF-α induced cellular toxicity and cell-death inducing signals was evaluated. Both peptides inhibited TNF-α-induced cell death and significantly down-
regulated the production and activity of caspase-3 and -7. Both peptides at all
concentrations tested failed to cause any damage to the treated C-20/A4
chondrocytes and additionally caused 40 - 50 % reduction in cleaved caspase-3
protein expression as determined by western blot. These results were confirmed
by testing the activity of the executioner caspases 3 and 7 and measuring
mitochondrial functionality following pre-treatment with α-MSH and D[TRP]β-γ-
MSH on TNF-α-activated C-20/A4 chondrocytes. The peptides exhibited strong
concentration-dependent protective effect against TNF-α-induced cell death,
whereby 49 % and 55 % reduction in chondrocyte apoptosis was observed
following treatment with α-MSH (3.0 µg/ml) and D[TRP]β-γ-MSH respectively.

Other studies support the molecular mechanism by which melanocortin peptides
prevent apoptosis in chondrocytes, with one particular study on neuronal cell-line
GT1-I demonstrating the inhibitory effect of the melanocortin peptide NDP-MSH
on caspase-3 activation as readout of apoptosis (Windebank et al., 1994) whilst
α-MSH prevents LPS/INF-γ-induced astrocyte apoptosis via activation of MC4
(Caruso et al., 2007).

To our knowledge, this study is the first to show the inhibitory effect of α-MSH
and D[TRP]β-γ-MSH in a model of TNF-α-induced chondrocyte apoptosis in vitro.
Through antagonism of MC3/4, demonstrates that MC1 and MC3 are involved in
the transmission of the anti-apoptotic effects of α-MSH and D[TRP]β-γ-MSH
respectively in the human C-20/A4 chondrocytic cell line. SHU9119 (10.0 µg/ml)
antagonized the effects of D[TRP]β-γ-MSH, but not α-MSH on down-regulating
the production of cleaved caspase-3 as a marker of apoptosis and consistently
reversed the protective effect of the selective MC3 agonist on mitochondrial
function in the model of TNF-α-induced chondrocyte death. The combination led
to 27 % up-regulation of cleaved caspase-3 production and cell death was
observed at TNF-α-produced levels regardless of the concentration used.

Combined, these results demonstrate, that MC3 is particularly important in
transmitting the anti-inflammatory, cyto-protective, anti-apoptotic and immuno-
modulatory effects of melanocortin peptides. In addition, the role of MC1 has
been also confirmed, given the fact that MC5 is non-functional in this C-20/A4
chondrocyte model and MC2 is solely activated by ACTH1-39 (Getting, 2006).
Effect of hypotonic solution on chondrocyte function.

To further understand the potential role that melanocortin peptides play in modulating chondrocyte activity, their effect was investigated on the function of osmotically challenged chondrocytes. Articular cartilage is highly hydrated tissue whereby approximately 30% of the water in the cartilage is found within the collagen intrafibrillar space (Hall, 1998). The amount of water in the cartilage depends on the fixed charge density of the proteoglycans, which bear strong negative electrical charges (Maroudas et al., 1979), neutralised by positive ions in the surrounding fluid. The high concentration of ions in the extracellular matrix, compared to the outside the tissue, has been shown to increase osmotic pressure (Maroudas, 1979; Maroudas and Evans, 1972). In OA and upon matrix degradation, the water content of cartilage increases and leads to over-hydration of the negatively charged proteoglycans, which alters the chondrocyte extracellular physio-chemical environment by reducing the osmolality causing an increase in cell volume; an early event during osteoarthritis (Gardner, 1992; Bush & Hall, 2004).

C-20/A4 chondrocytes were subjected to hypotonic conditions (280 to 140 mOsm), and a significant time-dependent up-regulation of pro-inflammatory cytokines IL-6 and IL-8 from C-20/A4 chondrocytes was observed, with a 37-fold increase in IL-6 and 8-fold up-regulation of IL-8 expression at 24 h and 42-fold and 10 fold, respectively at 72 h. These results were further substantiated by ELISA detection of these cytokines, which confirmed that the intensification on cytokine transcription, in response to chronic hypo-osmotic challenge, was translated into protein released from the C-20/A4 chondrocytes.

This study, to our knowledge, is the first to investigate the effect of lowered media osmolarity on C-20/A4 chondrocyte inflammatory profile. A novel finding, demonstrated by this work, is that upon reduction of extracellular osmolarity, C-20/A4 chondrocytes respond by increasing not only pro-inflammatory cytokines, but also the expression of MMP1 and MMP13, which increased in a time-dependent manner. MMP1 mRNA was up-regulated 2.3-fold following 24 h incubation in the 140 mOsm DMEM and this increased to 4-fold, compared to cells incubated in normal 280 mOsm culture media. Interestingly, MMP13 mRNA
(which is not present in non-treated C-20/A4 or healthy human cartilage) was elevated following stimulation with 140 mOsm media, and the detected amounts were significantly higher compared to TNF-α-stimulated levels. Hypo-tonicity did not seem to affect MMP3 expression in the first 24 h of incubation, but caused increased transcription when the chondrocytes were incubated for 72 h. This finding is supported by work on other cell type showing that cellular stresses such as pro-inflammatory cytokines and osmotic stress activate MAPK pathways (JNK, ERK1/2 and p38) (Lewis, 1998; Garrington, 1999). It has been shown that JNK and ERKs phosphorylate AP-1 family member c-Jun (Karin, 1995, Leppa et al., 1998), which then dimerizes with c-Fos and initiates the transcription of various MMP genes. Other groups have shown that the ERK1/2 pathway mediates the activation of the MMP1 promoter via an AP-1 element (Frost, 1994; Rutter, 1995; Korzus, 1997).

However, whether the altered osmolality of the C-20/A4 chondrocytes media is directly triggering the expression of these MMPs cannot be concluded by this work, as 140 mOsm DMEM also affects the synthesis of various cytokines, such as IL-6, which in turn can directly alter both MMP1 and MMP13 gene expression in a concentration-dependent manner. This is an important finding, since osteoarthritic cartilage is also defined by increased expression and synthesis of pro-inflammatory cytokines, matrix metalloproteinases and increased tissue hydration.

Since the events of OA, which we attempted to mimic here in the C-20/A4 chondrocyte model (increased production and release of pro-inflammatory cytokines and catabolic matrix metalloproteinases, increased degradation and reduced collagen type II production, chondrocyte apoptosis and cartilage hydration), it seems likely that a perpetuation of self-inducible and pathological events could lead to the chronic profile of this joint disorder, thus novel avenues for pharmacological intervention could look at targeting these processes.

The effect of α-MSH and D[TRP]²-γ-MSH on hypo-tonicity induced over-expression of matrix metalloproteinases was determined. D[TRP]²-γ-MSH down-regulated both MMP1 and MMP13 expression even at 24 h post stimulation with 140 mOsm DMEM. However, α-MSH only caused a modest non-significant
decrease in the transcription of \textit{MMP1} and \textit{MMP13}, its effect was not statistically different from the levels caused by the hypotonic media alone. None of the peptides altered the expression of \textit{MMP3} by C-20/A4 chondrocytes.

These results suggest, that \(\alpha\)-MSH probably due to its short half-life (6 h) is unable to down-regulate MMPs expression at 24 h, especially, since the hypo-osmotic medium, surrounding the chondrocytes seems to be exerting strong pro-inflammatory effects, which is unlike the effect of the pro-inflammatory stimuli used for various cell stimulation. The fact that IL-6 and IL-8 levels are increasing even at 72 h post incubation of C-20/A4 chondrocytes with 140 mOsm DMEM, suggested a possible role for these cytokines in the synergistic induction of MMPs expression at time-points as late as 24 and 72 h.

\textbf{Primary bovine chondrocyte (P\textsubscript{0}) activation by various pro-inflammatory stimuli.}

Following the identification of the protective role that melanocortin peptides could play on pro-inflammatory cytokines and MMP expression following TNF−\(\alpha\) stimulation in C-20/A4 chondrocytes, it was decided to determine whether these effects translated to primary cells and cartilage. C-20/A4 chondrocytes have been shown throughout this thesis to respond to various stimuli by secreting significant amounts of pro-inflammatory cytokines, chemokines and other non-cytokine pro-inflammatory mediators, such as NO and matrix metalloproteinases.

Even though immortalized cell lines are a suitable method for studying the function and the response of chondrocytes to various stimuli, primary cultures of articular chondrocytes may respond differently to these stimuli. In order to elucidate whether the findings confirmed in the C-20/A4 cell-line chondrocytes are consistent with the responses detected within primary chondrocytes, we employed primary articular chondrocytes extracted from load bearing regions of bovine knee joint cartilage.

High-density primary articular chondrocytes cultures (passage 0, P\textsubscript{0}) were established to study the effect of TNF-\(\alpha\) and LPS on the release of pro-inflammatory cytokines IL-1\(\beta\), IL-6, IL-8 and MCP-1. Primary chondrocytes reacted to both TNF-\(\alpha\) and LPS via a concentration-dependent up-regulation of
the production and release of all cytokines measured. TNF-α at all concentrations induced significant IL-6 release, which was highly comparable to the response observed in C-20/A4 cells, with the exception of the highest concentration of TNF-α (which was less effective in C-20/A4 compared to P₀ primary chondrocytes). These results are in accordance with previous observations that IL-6 mRNA is up-regulated following TNF-α-stimulation of human articular chondrocytes (Shlopov et al., 2000), or in OA tissue (Hrycaj et al., 1995, Shinmei et al., 1991). In addition, LPS was similarly potent in the induction of IL-6 secretion from both C-20/A4 cells and primary articular chondrocytes, despite somewhat higher levels in C-20/A4 cells.

In addition, bovine articular chondrocytes, much like the C-20/A4 chondrocytes were readily inducible to release IL-8, which can promote neutrophil-mediated inflammation and cartilage degeneration. TNFα and LPS promoted abundant IL-8 secretion from the primary bovine chondrocytes, a finding that is in agreement with the ability of these inflammogens to exert the same effect in human articular cartilage (Terkeltaub et al., 1991). The maximal IL-8 release from bovine chondrocytes was comparatively low, reaching just 50.0 pg/ml (TNF-α 40.0 – 60.0 pg/ml), as opposed to 205 pg/ml of IL-8 detected in C-20/A4 chondrocytes following stimulation with the same concentrations of the cytokine. Regardless of these slight discrepancies, this study accentuates the fact that articular chondrocytes are readily inducible to express the IL-8 gene and secrete biologically active IL-8, which can promote neutrophil-mediated inflammation and cartilage destruction.

TNF-α and LPS stimulated articular chondrocytes released significant amounts MCP-1, in concentrations concordant with the detected levels in C-20/A4 chondrocytes (maximum 120 pg/ml) following 6 h stimulation, thus confirming our initial findings. In agreement with the results of this study is the work by Lotz and colleagues, who have demonstrated the ability of human articular cartilage to respond to IL-1β stimulation by significantly up-regulating the transcription of MCP1 within 2 – 4 h of stimulation (Villiger et al., 1992). The fact that chondrocytes release biologically active MCP-1 in response to factors that are present in cartilage or synovium of osteoarthritic patients provides a possible
mechanism by which chondrocytes implicate in the initiation and progression of cartilage degradation observed in osteoarthritis.

IL-1β has a fundamental function in osteoarthritis pathophysiology as it controls the degeneration of articular cartilage matrix and severely affects chondrocyte apoptosis. Therefore targeting the activation mechanism of this catabolic cytokine seems to be essential as a therapeutic approach (Moldovan et al., 2000; Lopez-Armada et al., 2006; Kapoor et al., 2011). In this study, we have already demonstrated that both TNF-α and LPS are able to induce moderate secretion of this cytokine from human C-20/A4 chondrocytes. These findings were further substantiated by the detection of IL-1β secretion in response to TNF-α and LPS from primary articular chondrocytes as well. In both cell types, TNF-α generally initiated low (up to 40.0 pg/ml) levels of IL-1β secretion.

As previously discussed, the expression and secretion of all these pro-inflammatory cytokines has been previously detected in chondrocytes extracted from osteoarthritic cartilage (Martel-Pelletier et al., 1999; Maccoux et al., 2007; Villiger et al., 1992), which shows the relevance of the results acquired by the present study.

**Effect of melanocortin peptides on TNF-α-activated primary bovine articular chondrocytes.**

During this study, we have shown that human C-20/A4 chondrocyte express functionally active melanocortin receptors MC₁ and MC₃, and show very slight expression of MC₅, which was found to be functionally inactive. Here we demonstrate the expression of MC₁ in bovine primary chondrocytes and yet again we detected MC₅ expression. The expression of MC₃ unfortunately, was not investigated, because at the time the experiments were conducted the full sequence of the bovine MC₃ gene was not yet discovered, preventing the construction of primers.

Here, bovine articular chondrocytes consistently express MC₁, which is in agreement with other studies reporting the expression of this receptor in chondrocyte cell lines as well as primary cells (Yoon et al., 2008; Grässel et al., 2009). MC₅, which has also been detected, showed great variation between the
tested animals, with some samples showing slight expression, and others none at all. Additional work is required to fully unravel the expression of this melanocortin receptor in bovine primary chondrocytes.

To support our RT-PCR detection of the melanocortin receptors, we evaluated the pan agonist α-MSH, to test MC₁ functional activity, whilst the synthetic selective MC₃ agonist D[TRP]₈₋γ-MSH was used to evaluate whether bovine chondrocytes possess a functionally active MC₃. The MC₃/₄ antagonist and potent MC₁ agonist SHU9119 was also evaluated to determine 1) to determine whether D[TRP]₈₋γ-MSH specifically activates its putative receptor (MC₃) by blocking its binding sites; and 2) to conclude the role of α-MSH and MC₁ in modulation pro-inflammatory cytokines levels in the primary articular chondrocytes (P₀).

Pre-treatment of primary chondrocytes with α-MSH and D[TRP]₈₋γ-MSH prior to stimulation with TNF-α caused a concentration-dependent decrease in IL-1β a similar observation to that noted in the cell-line. However, a note should be taken that the inhibitory effect of α-MSH (1.0 and 3.0 µg/ml) on IL-1β concentrations (~40 % reduction) was lower in bovine chondrocytes compared to the cell-line, where similar concentrations caused > 80 % reduction.

Interestingly, lower concentrations of SHU9119 (1.0 and 3.0 µg/ml) led to a low 20 % inhibition in IL-1β release from bovine chondrocytes, suggesting a potential role for MC₁. However, SHU9119 was not capable of reducing either IL-6 or IL-8 production at any of the concentrations tested, which is in agreement with our findings from the human C-20/A4 chondrocytic cell-line. Conversely, both α-MSH and D[TRP]₈₋γ-MSH led to marked concentration-dependent bell shaped decreases in both IL-6 and IL-8 production. Unlike the effect of α-MSH on IL-1β, 1.0 and 3.0 µg/ml of α-MSH abrogated IL-6 production from the cultured primary bovine chondrocytes, by 93 % and 87 % reduction respectively. Similarly, D[TRP]₈₋γ-MSH (3.0 µg/ml) caused a similar degree of inhibition of IL-6, whilst both peptides inhibited IL-8 release, a similar observation as noted in the cell-line. This observation of bell-shaped inhibition of cytokine production by melanocortin peptides from primary cells was initially observed in macrophages following MSU crystal stimulation (Getting et al., 1999)
The MC<sub>3/4</sub> antagonist SHU9119, did not significantly alter the anti-inflammatory effect elicited by α-MSH on TNF-α-induced IL-1β, IL-6 and IL-8 production. Interestingly, SHU9119 and α-MSH (30.0 µg/ml) appeared to be synergistically acting to reduce the production of IL-6. From the results, it seemed that, with increasing concentrations of α-MSH, it started to slowly lose its effectiveness, and thus a combination with SHU9119 allowed it to “help” the α-MSH (30.0 µg/ml) to maintain the same level of cytokine inhibition. These findings are controversial, and may suggest that: 1) α-MSH does not act through activation of MC<sub>3</sub> in chondrocytes; 2) SHU9119, being relatively selective MC<sub>5</sub> agonist might be causing very mild reduction in IL-6 levels, only if high concentrations of α-MSH are used. This might be a resulting from desensitization of the receptors on chondrocytes and possible internalisation of MC<sub>1</sub>, allowing for or inducing a switch in the mechanisms by which the peptides works. It has been long known that GPCRs, such as melanocortin receptors, are regulated via multiple mechanisms (Clark, 1986, Perkins, 1991). This has been well illustrated by studies of β2-adrenergic receptor, reviewed in detail (Carman and Benovic, 1998, Ferguson <i>et al.</i>, 1998, Lefkowitz <i>et al.</i>, 1998), where agonist-induced activation of the receptor activates adenylate cyclase within seconds of binding (von Zastrow and Kobilka, 1992). However, following prolonged activation of the receptors, their ability to induce cAMP formation declined significantly. In addition, many GPCRs can be regulated by ligand-induced endocytosis or internalization (von Zastrow and Kobilka, 1992). SHU9119 (10.0 µg/ml) potently blocked and reversed the anti-inflammatory effect of D[TRP]<sup>5</sup>-γ-MSH at all concentrations tested, thereby suggesting that MC<sub>3</sub> receptor is expressed and functionally active in bovine primary chondrocytes.

Given the ability of these peptides to modulate pro-inflammatory cytokines, the peptides were evaluated on production of pro-resolving anti-inflammatory cytokines. IL-10 production was observed from stimulated bovine primary chondrocytes and is in agreement with that observed in human chondrocytes from healthy and osteoarthritic cartilage, which express both IL-10 and its putative receptor IL10R (Iannone <i>et al.</i>, 2001). Consistent with previous findings α-MSH caused the release of IL-10 (Bhardwaj <i>et al.</i>, 1996), the melanocortin peptides substantially elevated the production of the anti-inflammatory cytokine.
Primary bovine chondrocytes did not produce detectable basal IL-10 release; however, following 30 min pre-treatment of TNF-α-activated primary chondrocytes, there was a bell-shaped elevation of this cytokine following stimulation. Once again, SHU9119 failed to block the effect of α-MSH but completely abolished the anti-inflammatory effect of D[TRP]8-γ-MSH, therefore indicating a dual role for both MC1 and MC3 in modulating the inflammatory response in these cells.

**Cartilage impact and the anti-inflammatory and protective effect of melanocortins in models of mechanical trauma on cartilage tissue.**

Cartilage metabolism is contingent in part with mechanical forces, including shear stress and hydrostatic pressure that occur during normal joint loading. Previous *in vitro* work confirms that chondrocytes in culture continue to respond to a variety of loading conditions (Mankin and Lippiello, 1970). The distinctive properties of cartilage are believed to affect the chondrocyte reaction to pressure. It is largely documented that within physiological limits mechanical loading of healthy joints contributes significantly to the maintenance of the articular cartilage ECM by chondrocytes, but the precise relationship between mechanical loading and chondrocyte metabolism is still vague (Saamanen et al., 1987). Mechanical loading above physiological ranges and/or frequency gives rise to substantial cartilage injury (Burton-Wurster et al., 1993; Guilak et al., 1994) including increase in synthesis and release of pro-inflammatory mediators and degradative enzymes (Pickvance et al., 1993, Guilak et al., 1994, Wang et al., 2010b). Alteration in joint loading is additionally considered an important factor in the initiation of osteoarthritis (Anderson and Felson, 1988).

The effect of mechanical trauma on the functionality and metabolism of chondrocytes is receiving increasing attention (D'Lima, 2001a,b; Borrelli, 2004; Kurz, 2004), particularly because within mature articular cartilage, chondrocytes do not undergo cell division (Sailor, 1996; Buckwalter, 1998). Additionally, osteoarthritis is featured by reduced cellularity (Stockwell, 1991), a fact that is thought to contribute to the inability of the remaining chondrocytes to maintain normal matrix synthesis, thereby contributing to cartilage degradation (Bush et al., 2005).
Given the importance of this in the development of OA, the effect of a single impact blunt mechanical impact, delivered via drop tower (Bush et al., 2005) on chondrocyte viability and rates of pro-inflammatory mediator synthesis were determined. Following impact in situ chondrocytes within the superficial zone were shown to be particularly sensitive to mechanical injury confirming previous observations using bovine articular cartilage explants (Bush et al., 2005). Chondrocyte cell death was apparent 6 h post impact and was visibly localized to the distinct areas of impact. Confocal laser scanning microscopy and imaging allowed us to study the cell viability of individual chondrocytes within bovine articular cartilage explants subjected to a single blunt impact.

The data showed that ~3 % of the chondrocytes in non-impacted cartilage were non-viable following excision of the explant from the joint and increased significantly to 13 % following application of mechanical injury to the cartilage explant. The experiments conducted in this study were performed on cartilage explants excised from the underlying bone. However, judging from the results and the confinement of chondrocyte damage to the superficial zone, it is unlikely that the subchondral bone is a major factor of cartilage surface properties within these experiments (Bush et al., 2005).

It is important to note that this study has not been designed to clarify in vivo response to single blunt impact. The drop tower is a useful technique for simulating true impact (Bush et al., 2005), but it is not possible to mimic the impact waveform or displacement applied to the cartilage explant during compression. Nevertheless, the observed changes in matrix structure, surface damage to cartilage explant and the loss of chondrocytes in the superficial zone (Quinn et al., 1998, Quinn et al., 1999) are similar to the changes observed in osteoarthritic cartilage (Thompson et al., 1991; Wilder et al., 2002). Within this study, we have demonstrated the protective properties of melanocortin peptides in a model of TNF-α-induced chondrocyte apoptosis/cell-death. Here, we have shown that α-MSH (3.0 µg/ml), but not D[TRP]8-γ-MSH or dexamethasone (1.0 µM) are able to inhibit basal chondrocyte apoptosis in non-impacted cartilage. The melanocortin receptor pan-agonist decreased chondrocyte cell-death from 3 % to <1 %.
In order to expand our study on the effect melanocortins on primary chondrocytes in situ, we detected basal levels of pro-inflammatory mediator release of IL-1β, IL-6 and IL-8 in non-impacted cartilage and compared them to the effect of melanocortin peptides and dexamethasone. Our data demonstrates that chondrocytes from non-impacted cartilage produce significant basal levels of IL-1β, IL-6 and IL-8 cytokines, consistent with the effect of TNF-α on the chondrocytic cell-line. Previously, freshly isolated chondrocytes have been shown to spontaneously release detectable amounts of IL-8, but these are rapidly increased following stimulation, suggesting that functionally expressed chemokines by chondrocytes may trigger the release of matrix degradative enzymes and subsequent cartilage destruction (Lotz et al., 1992). IL-1β was also detected in cartilage explants prior to impact, similarly to that observed in normal human articular cartilage (Middleton et al., 1996). Bovine articular chondrocytes in situ also produced IL-6, a potent pleiotropic cytokine and important mediator of the cell interactions in osteoarthritis. This was consistent with the finding of Shinmei and collegues, who detected the expression of this cytokine in human articular chondrocytes (Shinmei et al., 1989). These basal release of cytokines were not modified by α-MSH, D[TRP]8-γ-MSH or dexamethasone.

However, the peptides led to significant reduction of cell death when administered 30 min prior to single blunt impact inflicted by the drop tower. At 6 h post-impact, α-MSH and D[TRP]8-γ-MSH caused significant protection of the chondrocytes from cell death, compared to levels detected following mechanical trauma in the absence of the peptide. Dexamethasone also caused a protective effect following impact although with a lower level of protection being observed compared to the peptides. Consistent with our previous observations that melanocortins can protect against TNF-α-induced chondrocyte apoptosis, these data confirms that melanocortins exert cyto-protective properties not only in cell-line chondrocytes in vitro, but also in primary articular chondrocytes in situ.

Mechanical injury was associated with a significant upregulation of IL-1β, IL-6 and IL-8 release compared to levels produced by chondrocytes in non-impacted cartilage, and the melanocortin peptides α-MSH, D[TRP]8-γ-MSH and dexamethasone significantly down-regulated the release of these cytokines. α-
MSH was the least effective of the three treatments on cytokine release, whilst D[TRP]³-γ-MSH was more effective than α-MSH in reducing these cytokines levels. Dexamethasone showed the greatest effect in reducing pro-inflammatory synthesis caused by mechanical trauma to the cartilage explants. Here, in accordance with our previous results, melanocortins exhibited modulatory effects on the production of pro-inflammatory cytokines from impacted cartilage, rather than complete abrogation of the cytokine response as previously seen in models of acute inflammation (Getting et al., 1999, 2002, 2006, 2008). However, the data for dexamethasone was contradictory to the previous findings, showing that dexamethasone abrogated completely the release of these cytokines from cell-line chondrocytes. One explanation for these findings might be the relatively low permeability of the cartilage matrix, compared to culture media, which poses no physical barrier for the drugs to reach and affect chondrocytes and their function. Furthermore, pro-inflammatory mediator release from C-20/A4 cell line was caused by exogenous TNF-α-stimulation, whereas in situ articular chondrocytes were activated by mecanical trauma. Nevertheless, it is important to note that joint trauma leads to excessive synthesis of pro-inflammatory cytokines, one of the major ones being TNF-α (Ertel et al., 1995), which could potentiate the cartilage destruction shown by our work on C-20/A4 chondrocytes.
Chapter 5
Conclusion and Future work
5.1 Conclusion.

The results generated in this thesis indicate that C-20/A4 chondrocytes and primary articular chondrocytes in vitro and in situ respond to exogenous stress (pro-inflammatory mediators, changes in extracellular osmolarity, mechanical trauma) by producing significant amounts of pro-inflammatory cytokines (such as IL-1β, IL-6, IL-8 and MCP-1), and collagen derisive matrix metalloproteinases (MMP1 and MMP13), therefore corroborating the possible role of the chondrocyte in initiation and progression of cartilage degradation, as observed in OA. In addition, the work presented here indicates that there is significant down regulation of cartilage ECM components such as collagen type I and type II upon TNF-α-activation of C-20/A4 chondrocytes, which if translated to in vivo work could additionally lead to a reduction in the quality of the cartilage matrix, thereby leaving the cartilage prone to injury.

Melanocortins have been previously shown to possess a vast range of physiological and pharmacological actions. However, little research has occurred with respect to their role in chondrocyte inflammation. This thesis highlights the anti-inflammatory and anti-apoptotic effects of melanocortin peptides α-MSH and D[TRP]8-γ-MSH in an in vitro chondrocyte-based model of TNF-α-induced stimulation. The melanocortin peptides were able significantly down regulate pro-inflammatory cytokines and cartilage degradative MMPs, but also to lower cytokine-induced apoptosis and to induce the anti-inflammatory proteins (IL-10 and HO-1) in this cell-line. These effects were transmitted through the activation of MC1 and MC3, both of which were found to be expressed and functionally active in C-20/A4 chondrocytes. The influence of the peptides was not limited to the human cell-line chondrocytes; instead, the results highlight the ability of the peptides to reduce TNF-α-induced pro-inflammatory cytokine and MMP expression in primary bovine articular chondrocytes.

Moreover, the data presented here confirm that mechanical impact is an effective modulator of chondrocyte metabolism in vitro. This information adds to our basic understanding of how mechanical loading influences articular cartilage metabolism. Understanding the precise mechanisms by which impact trauma alters cartilage metabolism will provide vital insights for development of
approaches for the treatment of arthritic pathologies. The melanocortin peptides not only showed chondroprotective effect in C-20/A4 chondrocytes, but also in articular chondrocytes in situ. This is a novel finding, suggesting potential clinical implications whereby people with joint injury/trauma or people suffering from OA could benefit from potential melanocortin peptide treatment.
In the presence of TNF-α, Mechanical Injury, or change in ECM osmolarity, Chondrocytes express functional MCR 1 and 3 upon binding of melanocortin peptides to the MCR.
Figure 5.1 Melanocortins modulate pro-inflammatory cytokine production and exhort anti-inflammatory and cytoprotective properties in chondrocytes.

The binding of melanocortins to either MC1 or MC3, functionally active on the membrane of chondrocytes, increases cAMP, which activates protein kinase A (PKA), therefore leading to four main effects. First, PKA activation induces the phosphorylation of the cAMP-responsive-element-binding protein (CREB) prevents the association of CBP with p65. b) The activated PKA hinders IκB kinase (IKK), which stabilizes the IκB inhibitor and stops nuclear translocation of NF-κB. c) PKA activation prevents MAPK/ERK kinase kinase 1 (MEKK1) phosphorylation and activation, and activation of p38 and TATA-binding protein (TBP). Non-phosphorylated TBP is unable to bind to the TATA box and to form dynamic trans-activating complex with CBP and NF-κB. A reduction in the amounts of nuclear p65, CBP and phosphorylated TBP inhibits the formation of the conformationally active trans-activating complex that is required for the transcription of most cytokine and chemokine genes, therefore a great reduction in the synthesis of IL-1β, IL-6, IL-8 and MCP-1 by the chondrocyte is observed. d) Fourth, inhibition of MEKK1 deactivates JUN kinase (JNK) and cJUN phosphorylation. The composition of the activator protein 1 (AP1) complex changes from the transcriptionally active cJun–cJun, to the transcriptionally inactive cJun–cFos or CREB. In addition, melanocortin peptides inhibit chondrocyte apoptosis by significantly inhibiting TNF-α-induced caspase-3 production and deactivating both executioner caspases - caspase-3 and caspase-7. Furthermore, activation of MC1 and MC3 led to significant up-regulation of production of IL-10 and HO-1, which support and assist the resolution of inflammation.

The final consequence is that the transcriptional machinery of chondrocytes is significantly disrupted by the melanocortin treatment, thereby driving pro-resolution of inflammation and emphasizing the anti-inflammatory and cytoprotective properties of the melanocortin peptides. This study exploits the melanocortin pathways as a first step towards possible development of future anti-inflammatory therapeutics and emphasized the need of further research in this direction.
5.2 Future work

- To investigate the effect of melanocortin peptides on collagen production by activated/osteoarthritic chondrocytes.

- To localize *IL-6*, *IL-8*, *MMP1* and *MMP13* gene loci to either euchromatin or heterochromatin in healthy and osteoarthritic chondrocytes and to investigate the effect of selective melanocortin peptides on the localization of these gene loci.

- To scrutinize the mechanism of hyper- and hypomethylation of *MMP1*, *MMP3* and *MMP13* following stimulated/osteoarthritic chondrocytes; to evaluate how epigenetic changes may relate to the pathogenesis of osteoarthritis.

- To investigate the effect of melanocortin treatment on cytokine and MMP genes methylation status in normal and osteoarthritic chondrocytes.

- To evaluate the peptides in pre-clinical models of OA and to look at the effect of these peptides on human tissue.
Chapter 6
References


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Appendix

Publications