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PROTEASOME TARGETED THERAPY IN ARTHRITIS MODELS

-EFFECTS ON PAIN AND INFLAMMATION

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To my family

ABSTRACT

The ubiquitin proteasome system (UPS) regulates numerous cellular functions by selectively degrading cellular proteins. It controls activation of the transcription factor NF-κB which is involved in the expression of pro-inflammatory mediators. The UPS regulates neuronal signalling by controlling levels of synaptic proteins during chronic pain.

Pain and inflammation are the major components of inflammatory joint disorders. The focus of this thesis has been to further investigate mediators of pain and inflammation and to explore the role of UPS in pathogenesis of chronic arthritic conditions such as the rat models of rheumatoid arthritis (RA) and osteoarthritis (OA).

Opioid peptides dynorphins are involved in the maintenance of pathological pain. The localization and distribution pattern of dynorphins precursor protein prodynorphin (PDYN) was studied in the brain. We could demonstrate that high levels of PDYN were present in the amygdala, hippocampus and stratum and lower amounts in the cerebral cortex. Furthermore, PDYN was detected in the ventral trigeminal area and in the hypocampal CA3 regions that do not have cell bodies of PDYN-producing neurons but contain axons and axon terminals. PDYN is thus transported to and stored in axon terminals prior to release from secretary granules as mature peptides. Furthermore, we could demonstrate that the depolarization of neuronal cells stimulates processing of PDYN into mature dynorphins which may represent the local regulation of synaptic transmission.

The UPS regulates the processing and secretion of dynorphins and CGRP during neuropathic pain. We investigated the role of UPS in pain and inflammation in adjuvant arthritis by the using proteasome inhibitor MG132. We demonstrated an increased expression of p50 (a subunit of NF-κB) and NF-κB activity in arthritic joints. A decrease in p50 expression and in NF-κB activity was observed and coincided with reduced arthritic severity when MG132 was administered. Furthermore, an increased expression of SP and CGRP was observed in arthritic joints to correspond with increased pain. MG132 therapy reversed the up-regulated expression of SP and CGRP in the arthritic joints and of SP and PDYN in both the dorsal root ganglia (DRG) and in the spinal cord (SC). These results suggest that the UPS regulates pain and inflammation at the peripheral tissues. UPS-mediated protein regulation in the peripheral and central nervous system most likely also regulates inflammatory and nociceptive mediators.

In addition, the role of UPS in the mediation of pain and disease progression was studied in OA. Pain mechanisms in OA are unclear but the sensitization of nociceptors in the synovium and bone probably contributes to the initiation and maintenance of pain. We demonstrated that the decreased pain thresholds were related to the increased expression of SP and CGRP in the knee joint and in corresponding DRG. We observed an enhanced expression of matrix metalloproteinase-3 (MMP-3) in the knee joints that coincided with pathological changes in the OA cartilage. MG132 administration caused a significant reversal of pain behavior, attenuated cartilage and bone destruction and resulted in a decrease in SP, CGRP and MMP-3 expression in knee joint.

In conclusion, the UPS represents a major intracellular pathway that critically regulates the development of both joint inflammation and inflammatory pain. In future perspectives, novel safe proteasome inhibitors with limited adverse side-effects would be available with the benefit of targeting both pain and inflammation.

LIST OF PUBLICATIONS

I. Yakovleva T, Bazov I, Cebers G, Marinova Z, AHMED A, Vlaskovska M, Hara Y, Johansson B, Hochgeschwender U, Singh I.N, Bruce-Keller A.J, Hurd Y.L, Terenius L, Ekström T.J, Hauser K.F, Pickel VM. and Bakalkin G. **Prodynorphin storage and processing in axon terminals and dendrites**
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II. AHMED A, Li J, Ahmed M, Hua L, Ossipov MH, Yakovleva T, Bakalkin G, Stark A. **Attenuation of pain and inflammation in adjuvant-induced arthritis by the Proteasome inhibitor MG132**
Arthritis Rheum. 2010; 62(7):2160-9

III. AHMED A, Ahmed M, Li J, Margarita D, Bakalkin G, Stark A, Harris, HE. **Substance P and Prodynorphin levels are modified by the Proteasome inhibitor MG132 in inflammatory pain**
Manuscript

IV. AHMED A, Li J, Harris, HE, Stark A, Bakalkin G, Ahmed M. **Proteasome inhibitor MG132 ameliorates the progression of experimental osteoarthritis in the rat**
Submitted

CONTENTS

LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
AIA	Adjuvant- induced arthritis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASICS	acid-sensing ion channels
Bcl-2	B-cell lymphoma 2
BMI	body mass index
BMP	Bone morphometric protein
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CTX-II	cartilage degradation marker-II
DMARD	disease modifying anti-rheumatic drugs
DMOAD	disease modifying osteoarthritic drugs
DRG	dorsal root ganglia
DUB	deubiquitinated enzymes
FDA	Food and Drug Administration
HHGS	Histological-Histochemical Grading System
IAP-1	Inhibitor of Apoptosis Protein-1
IκB	Inhibitory kinase B
IL-1	interleukin-1
MHC	major histocompatibility complex
MM	multiple myeloma
MMPs	matrix metalloproteinases
NF-κB	Nuclear Factor-kappa B
NHL	non-Hodgkin lymphomas
NKA	neurokinin A
NMDA	N-Methyl-D-aspartate
NO	nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
PDYN	prodynorphin
PTPN22	protein tyrosine phosphatase non-receptor type 22
PWT	paw withdrawal threshold
RA	rheumatoid arthritis
RANKL	Receptor Activator for Nuclear Factor κ B Ligand
RVM	rostral ventromedial medulla
SP	substance P
TNF-α	tumour necrosis factor alpha
Ub	ubiquitin
UPS	ubiquitin proteasome system
VDR	vitamin D receptors
VPL	ventral posterior lateral

1 INTRODUCTION

Due to demographic changes there is a continuous increase in the proportion of elderly people with disabling and degenerative disease. For the vast majority of these patients, pain is the predominant symptom and the main reason for disability, regardless of whether the underlying disease is rheumatoid arthritis, osteoarthritis, fibromyalgia, osteoporosis or skeletal metastasis. The mechanisms underlying pain are still not clear.

To date, the prevailing means of preventing and treating painful and inflammatory conditions have been unable to offer satisfactory solutions. Physiotherapy and surgery for being expensive and therapeutically of limited value are unable to meet the demands. Surgical interventions such as joint replacement are an effective treatment for joint destruction (1). One has to remember that joint replacement does not last forever and that revision surgery is very demanding (2). There are also several diseases such as fibromyalgia for which there is no indication for surgery. Pharmacotherapy, possibly in combination with the others, is presumably the one realistic option in resolving the problem in a wider perspective. Until now, Pharmacotherapy in chronic pain conditions, however, has only been useful for patients with slight-to-moderate symptoms, partly because of the side-effects at higher doses. Thus the patients in greatest need of pain relief, constituting a major proportion, are left without a viable alternative except for a small subset that is suitable for surgical intervention. There is therefore an urgent need of more effective pharmacotherapy.

The aim of this thesis work has been to identify mediators of pain and inflammation in inflammatory joint diseases and to study the effect of the reversible proteasome inhibitor MG132 on these mediators with special reference to rheumatoid arthritis (RA) and osteoarthritis (OA), two most common joint disorders, using animal models.

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune disease that primarily affects the joints. The functional disability that results from progressive joint destruction is associated with substantial cost, significant morbidity and premature mortality. RA affects about 0.5 -1% of the population. Women are affected 2-to-3 times more often than are men. Onset of the disease can be at any age but is most frequent in 35-to-50 year olds. RA has a worldwide distribution with a notable low incidence in Africa and Asia, and a high prevalence in Europe as well as in Native American populations (3, 4).

1.1.1 Etiology

Although RA involves autoimmune reactions, the precise cause is still unknown. Both genetic and environmental factors have been identified to play a role in RA susceptibility. The most important genetic risk factors are linked to the class II major histocompatibility complex (MHC) on chromosome 6 (5, 6) and to a non-MHC gene tyrosine phosphatase non-receptor type 22 (PTPN22), a gene that regulates T-cell activation and increases RA risk by 40-80% (7). Not only genetic but also environmental factors such as smoking, diet, and birth weight and socioeconomical status are some of the features identified as risk factors for RA (8-10). Combination of genetic factors along with smoking further increases the risk for RA (8).

1.1.2 Symptoms

In RA general disease features include fatigue, lack of appetite, low-grade fever, muscles and joint stiffness. In RA, multiple joints are involved which become swollen, painful and tender due to synovitis and destruction of cartilage and bone.
RA is clinically a heterogeneous disease, with no single diagnostic or pathological symptoms. Diagnostic criteria was developed by the American College of Rheumatology (ACR) in 1987 and used for clinical diagnosis of RA and also serve as the classification criteria for RA (Table 1). 4 out of 7 criteria should be fulfilled to establish the diagnosis of RA.

Table 1. The ACR classification criteria for RA

Morning stiffness for at least one hour and present for at least six weeks
Swelling of three or more joints for at least six weeks
Swelling of wrist, metacarpophalangeal, or proximal interphalangeal joints for at least six weeks
Symmetric joint swelling
Radiographic changes
Rheumatoid subcutaneous nodules
Rheumatoid factors

1.1.3 Pathophysiology of RA

RA is characterized as the disease of the synovial membrane. In normal joints the synovial membrane that lines the non-weight-bearing surface of joints is thin and comprises two layers and produces lubricating and nourishing synovial fluid. Within the synovia, resident cells are macrophage-like synoviocytes, fibroblasts-like synoviocytes, nerve fibres and adipocytes. During RA synoviocytes proliferate and inflammatory cells are recruited from the blood and synovial membrane thickened to 6-8 layers or more (Figure 1).

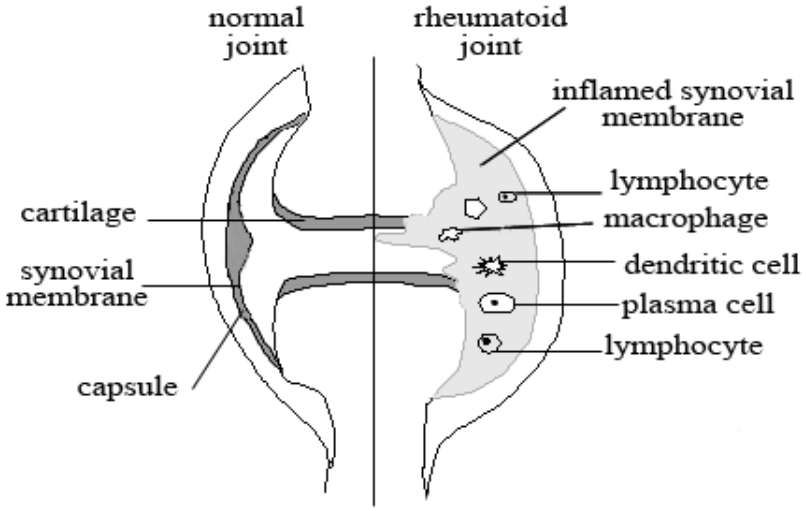

Figure 1. Pathophysiological changes in the RA joint

Macrophages play a crucial role in RA. Activated macrophages produce prostaglandins and other inflammatory mediators including IL-1 and TNF-α and up-regulate MHC-class II in response to stimuli. Plasma cells in inflamed synovial membrane produce large quantities of immunoglobins including autoantibodies such as RF. T lymphocytes are present within the deeper layers of the synovium. Activated T lymphocytes produce certain cytokines such as IL-17 which can be detected both in the synovial fluid and synovial membrane.
Cytokines are key players in pathogenesis of RA. Synovial fluid contains large quantities of cytokines secreted by macrophages, dendritic cells, neutrophils and synovial fibroblasts. Cytokines such as TNF-α, IL-1 and IL-17 stimulate the production of destructive proteases, Matrix metalloproteinases (MMPs) which break down collagen and have been implicated in joint destruction. The inflamed synovium forms a pannus tissue, which grows into the articular cartilage and cause bone destruction. Bone destruction first appears as marginal erosions at the site of synovial proliferation, where bone is unprotected by hyaline cartilage. Subsequent bone destruction leads to sublaxation and deformity.

1.1.4 Therapies

Therapies include NSAID drugs, systemic administration or intra-articular injections of corticosteroids, disease modifying anti-rheumatic drugs and specific antagonists against TNF-α or IL-1 provide efficient treatments to many but not to all RA patients (Table 2). The search for more beneficial therapies is still warranted.

Table 2. Therapies available for treatment of RA.

NSAIDs	Non-steroidal anti-inflammatory drugs. Analgesic and mild-anti-inflammatory effect. Do not alter the disease course or prevent joint destruction
Corticosteroids	Anti-inflammatory mechanism. Prevents joint damage.
DMARDs	Disease modifying anti-rheumatic drugs. Halt or retard progression of disease. Methotraxate, sulphasalazine, gold salt, chloroquine and cyclosporine A.
Biological	Antagonists including mAb and soluble receptors against endogenous proinflammatory mediators. TNF blocker (infliximab, etanercept, adalimumab), rIL-1 receptor antagonist, IL-6 R blocking mAb, anti-CD20 mAb.

1.2 OSTEOARTHRITIS

Osteoarthritis (OA) is the most common form of arthritis and a leading cause of chronic disability worldwide. OA can affect any joint but the most frequently affected sites are the hands, knees, hips, and spine (11). Clinically significant osteoarthritis of the knee, hand, or hip occurs in 8.9% of the adult population with knee OA as the most common type (6% of all adults) (12, 13). The prevalence of OA in middle-aged people causes society a considerable burden due to loss of working time and early retirement. OA is the most common reason for total knee and hip replacement (14).

Osteoarthritis is classified as being *primary* or *secondary*. Primary OA is age-related and is common among the older population. Constitutional factors are of importance for the development of primary OA. Medical conditions such as trauma, abnormally

formed joints, hip dysplasia, diabetes, and inflammatory chronic arthritis may cause development of secondary osteoarthritis. The symptoms are similar to those of primary osteoarthritis.

1.2.1 Etiology

A number of risk factors such as mechanical injury, obesity, peripheral neuropathy, genetic factors and increasing age have been identified, and interaction of these risk factors is known to initiate this musculoskeletal disorder but still the exact etiology is not known (14).

Obesity is considered as one of the most important risk factors for OA, especially for OA manifest in knees. High BMI (>30) is associated with knee OA but not with hip OA (15). Several studies indicate a role of genetic factors in an earlier onset of OA. Twin studies in females have revealed that the influence of genetic factors in hand OA may approach up to 70% (16). Candidate gene studies and genome-wide linkage analyses have revealed polymorphisms or mutations in genes encoding extracellular matrix molecules (type II collagen (COL9A1), Aggrecan (AGC1)) and signalling molecules (Bone morphometric protein (BMP5), vitamin D receptors (VDR)) (17, 18). In contrast to RA, no MHC class II associated gene has been identified.

The likelihood of developing osteoarthritis increases with age. The prevalence of having knee OA is negligible between 25-34 years of age and increases to 20-40% in those aged 75 and older (19, 20). The prevalence is higher in men than in women up to approximately age 45; after 45 it is reversed and by 65 years of age and over the female: male ratio ranges from 1.5:1 to 2:1 (11). Hip osteoarthritis is less common than knee osteoarthritis but symptoms are often severe. Population-based radiographic surveys of patients aged 55-74 revealed an increase in prevalence with age, with 16% in men and 6% in women (19, 21).

1.2.2 Symptoms

OA symptoms are often associated with significant functional impairment, as well as signs and symptoms of pain, inflammation, joint stiffness and deformity, and loss of mobility (13). These symptoms may develop in one or more joints. Pain symptoms are increased when the joint is loaded. With progression of disease the pain can occur at rest.

Diagnostic evaluation includes patient history, physical examination, radiological examination and in some cases laboratory testing. The clinical symptoms and signs of osteoarthritis and its radiological correlates follow a typical course of disease progression and can be incorporated into a clinically useful staging system. A number of different joint-specific scoring systems have been developed which differ from each other in subjective and objective criteria (22).

The Histological-Histochemical Grading System (HHGS) originally proposed by Mankin in 1971 is a commonly used grading system for evaluation of osteoarthritic cartilage (23). Originally developed for the assessment of human articular cartilage it has also been used for grading of animal cartilage destruction (22). Although frequently used the HHGS score has been criticized for its questionable reproducibility and inadequate assessment (24, 25). In 2006 the Osteoarthritis Research Society International (OARSI) developed an alternative Osteoarthritis Cartilage Histopathology Assessment System (26). The OARSI system emphasizes the extent of cartilage

damage over the articular surface through different stages over disease development, in addition to damage analyzed at several levels of the cartilage layer (26)

1.2.3 Pathophysiology

Recent advances in image techniques (MRI) indicate OA as a disease of the entire joint. The pathophysiology of OA involves not only the breakdown of articular cartilage but also changes in the subchondral bone and synovium (27). The cartilage has received most attention in past years due to extensive damage evident in pathology specimens and from imaging studies.

Human articular cartilage is continuously remodelled as a result of anabolic and catabolic processes. The chondrocytes in normal adult cartilage maintain a balance between synthesis and degradation of extracellular matrix components. The metabolic activity of the chondrocytes in OA is shifted toward a state where new matrix synthesis is outweighed by the breakdown of matrix constituents, with an increased production of catabolic cytokines and matrix-degrading proteinases. Loss of joint surface integrity occurs as a result, and cartilaginous vertical clefts develop (fibrillation), deeper lesions exposing the subchondral bone. The exposed bony surface can have necrotic lesions that lead to the formation of bone cysts. Simultaneously, angiogenesis of subchondral bone marrow as a result of the initial insult to the bone tissue causes calcification of the affected cartilage that stimulates endochondral ossification (osteophytes) (27, 28).

In addition to the articular cartilage the synovium will be infiltrated with inflammatory mediators. Ligaments that are often lax, and bridging muscles, become weak and the neuromuscular apparatus may also display pathophysiological changes. The classification of OA as a non-inflammatory process is in part due to the low synovial fluid leukocyte counts. In contrast, the clinical signs (i.e. swelling, effusions and stiffness) clearly reflect synovial inflammation. While environmental and genetic factors influence the incidence and progression of OA, the damage incurred by joint tissues is mediated by a variety of cytokines, growth factors, proteases and inflammatory mediators released by the chondrocytes and synoviocytes (Figure 2) (29, 30).

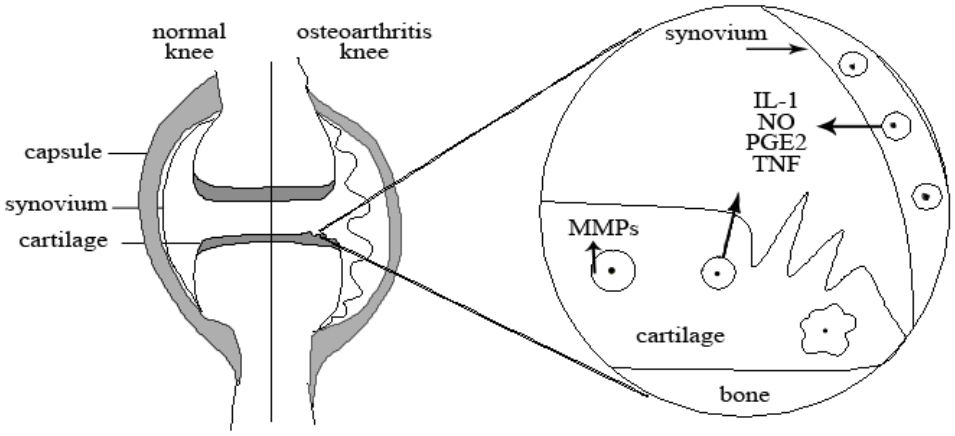

Figure 2. Pathophysiological changes in the osteoarthritic knee joint, modified from reference (27)

1.2.4 Therapies

Osteoarthritis is not a curable disease at present, as the mechanism by which it arises and progresses remains incompletely understood. The goal of treatment is therefore to alleviate the signs and symptoms of the disease and, if possible, to slow its progression. The therapeutic spectrum ranges from conservative treatments such as physiotherapy, orthopedic aids and orthosis, pharmacotherapy and finally surgery.

The most widely used drugs are NSAIDs which provide incomplete relief in chronic patients (31). A number of new agents, referred to as disease-modifying osteoarthritic drugs (DMOADs) are now the subject of preclinical and clinical trials (e.g. Risedronate (bisphosphonates)). Imaging data indicate that it fails to stop OA disease progression, despite improvement of symptoms and reduced cartilage degradation markers (such as CTX-II) in both a 1-year study (32) and a 2-year multinational phase III trial of 2400 knee OA patients (33). There is thus a need for more effective pharmacotherapies to mitigate disease progression as well as pain. The classes of medications used to treat osteoarthritis (Table 3).

Table 3. Agents Used to Treat Osteoarthritis (34)

Oral non-opioid analgesica (eg, Acetaminophen)
Topical NSAIDs (e.g. capsaicin cream)
NSAIDs
Intra-articular steroid injections
Opioid anelgesics

1.3 PAIN

Pain has been defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage (35). Mechanical, thermal or chemical damage to the tissue sensitizes pain receptors (nociceptors) on primary afferent neurons which transmit a signal along the spinal cord to the brain. This process involves a variety of autonomic responses and is called *nociception* and causes the perception of pain.

1.3.1 Types of pain

According to the pathogenesis, pain can be classified as:

1.3.1.1 Nociceptive pain

Nociceptive pain is initiated by the stimulation of nociceptors as a result of tissue injury and/or inflammation. Nociceptive pain can be further divided into *somatic* and *visceral* pain. Somatic pain can be s*uperficial or deep somatic* pain. *Superficial somatic* pain is initiated by the activation of nociceptors in the skin or superficial tissues. *Deep somatic* pain is initiated by the stimulation of nociceptors in ligaments, tendons, bones, blood vessels or in muscles. *Visceral* pain originates in the viscera (organs) and is extremely difficult to locate. Several visceral regions produce ‘referred’ pain when injured, where the sensation is located in an area distant from the site of injury or pathology.

1.3.1.2 Neuropathic pain

Neuropathic pain is caused by the injury or disease of neurons. It is defined as peripheral neuropathic pain if the injury is in the peripheral nervous system and central neuropathic pain if the neurons in the central nervous system are effected (36). In many cases pain is not strictly inflammatory or neuropathic because neuropathy may involve inflammatory components and neuropathic components may contribute to inflammatory pain states (37). Pain can be distinguished as being acute or chronic pain, the latter reflecting pain experienced in excess of 6 months (38).

1.3.2 Pain system

Pain transduction and perception is an extensive and complex process involving fundamental biological events at multiple levels of the nervous system. Noxious stimuli activate primary afferent neurons with free nerve endings. Primary afferent nerve fibres can be classified into three types on the basis of their diameter, structure and conduction velocity.

C-fiber C-fibers have unmyelinated axons with a diameter ranging from 0.4-1.2 μm, conduct action potentials slowly at a rate of 0.5 to 2.0 m/sec, and have small-diameter cell bodies. C-fibers mediate the slower, burning quality of pain. C-fibers comprise approximately 70% of all nociceptors. A variety of neuropeptides, including substance P (SP) and calcitonin gene-related peptide (CGRP) have been identified in C-fibers.

Aδ-fiber Aδ-fibers have lightly myelinated axons, with a diameter ranging from 2-6 μm, conducting nerve impulses at a rate of 12 to 30 m/sec, and have medium-sized cell bodies

Aβ-fiber Aβ-fibers have myelinated axons, with a diameter ranging > 10 μm, conducting nerve impulses at a rate of 30-1000 m/sec and have large-sized cell bodies. Aβ-fibers mediate the fast, pricking quality of pain.

Cell bodies of C- and A-fibre neurons are located within the peripheral sensory ganglia such as dorsal roots (DRG). DRG neurons have very long t-shaped axons with one end forming a sensory terminal at the skin or viscera and another end synapsing in the dorsal horn of the spinal cord. In the spinal cord these neurons project to the outermost region of the spinal dorsal horn (lamina I and outer lamina II) and terminate largely on spinal neurons that project to higher-order pain centers such as the cortex and the hypothalamus in the brain.

1.3.3 Mediators of pain

Several classes of neurotransmitters and neuromodulators present in the primary afferent fibres, spinal dorsal horn and in brain regions are suggested to involve in nociception. These include excitatory amino acids (glutamate and aspartate), sensory and autonomic neuropeptides (SP, CGRP, NKA, and CCK), prostaglandins, bradykinin, nitric oxide (NO) and adenosine in the primary afferent fibres (40-42). In the dorsal horn of the spinal cord, neurotransmitters (e.g., glutamate, SP and CGRP) from the primary afferent terminals and on the post-synaptic membrane, neuropeptides (e.g., SP, CGRP and dynorphins) and their receptors (e.g, glutamate receptors, NMDA, AMPA) participate in central sensitization (43, 44).

1.3.3.1 Sensory neuropeptides

Sensory neuropeptides, SP and CGRP are synthesized in neurons of dorsal root ganglia. Almost 90 % of SP is transported distally (45). Apart from it nociceptive role, SP has

also been found to participate in proinflammatory mechanisms. CGRP is often localized with SP in unmyelinated C fibre facilitates the release of SP at the spinal cord level and delay SP degradation and potentiate the nociceptive effect.

1.3.3.2 Dynorphins

dynorphin A, dynorphin B, and α-neoendorphin, collectively known as dynorphins are cleaved from the precursor protein prodynorphin (PDYN) (46). These peptides can be metabolized into shorter forms such as Leu-enkephalin-Arg^6 and Leu-enkephalin. Dynorphins have high affinity for κ-receptors. Dynorphin opioid peptides and their receptors exhibits wide spread distribution in mammalian brain and involved in numerous functions such as pain regulation, learning process, neuroendocrine function and modulation of stress. In many cases dynorphin has no direct action but modulates the action of other neurotransmitters such as SP. In the spinal cord dynorphins are present in the dorsal horn (47).

1.3.4 Pain mechanism

1.3.4.1 Ascending nociceptive pathway

A Noxious stimulus excites thin myelinated Aδ and unmyelinated C-fibres primary afferents (48) and induces release of neurotransmitters (e.g., glutamate and substance P) from the terminals of theses neurons in the laminae I, II or V of the dorsal horn. These released neurotransmitters induce excitation of post-synaptic membrane receptors (e.g, glutamate receptors) of the dorsal horn neurons (49, 50). The pain sensation is then transmitted through the spinothalamic tract to the thalamus and is then conveyed to various areas of the brain such as the cortex and the hypothalamus. The spinothalamic tract is mainly a crossed path, originating from neurons located in the dorsal horn Rexed´s lamina I and V and terminating in several different thalamic nuclei, including the ventral posterior lateral (VPL) nuclei (51).

1.3.4.2 Descending nociceptive pathway

From brainstem nuclei, impulses ‘descend’ into the spinal cord and influence the transmission of pain signals at the dorsal horn (52, 53). Concerning descending inhibition, the periaqueductal grey matter (PAG) is a key region. It projects to the rostral ventromedial medulla (RVM) and receives inputs from the hypothalamus, cortical regions and the limbic system (53). Neurons in the RVM then project to the dorsal horn and through motor neurons to the site of injury.
The immediate reaction to pain is transmitted over the reflex arc by sensory fibres in the dorsal horn of the spinal cord and by synapsing motor neurons in the anterior horn. This anatomical pattern of sensory and motor neurons allows the individual to move away quickly from the pain source.

1.3.5 Inflammatory joint pain

In inflammatory joint disorders such as RA and OA pain is generally perceived to arise from a combination of peripheral and central mechanisms, but emerging data indicate that peripheral inflammation involves complex neuroimmune interactions resulting in primary hyperalgesia. A large range of inflammatory molecules can directly sensitize nociceptors of primary afferent neurons (54, 55) which start to respond to light pressure and movements in the working range of the joint. Most of these units are thinly myelinated Aδ fibres or unmyelinated C fibres. Finally, initially mechano-insensitive fibres (silent nociceptors) become responsive to mechanical stimulation of the joint and

contribute to the afferent inflow into the spinal cord during inflammation (56, 57). Collectively, these changes provide the afferent sensory basis of joint pain. The consequence of these processes is that during inflammatory conditions the nociceptive system is activated by normally innocuous and non-painful mechanical stimuli.

Previous studies indicate that central and peripheral neuronal mechanisms play a role in inflammatory joint disorders, particularly with regard to inflammation and pain (58). Sensory neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) have been demonstrated to participate not only in pain modulation but also in inflammatory processes. Previously, an up-regulation of the SP and CGRP in the ankle joints and their corresponding dorsal root ganglia was demonstrated in adjuvant arthritis (59). The development and progress of joint inflammation in adjuvant arthritis was significantly attenuated by using the neurotoxin capsaicin, which specifically down regulates sensory innervation (60). The beneficial effects of capsaicin on joint inflammation were correlated with reduced levels of SP and CGRP in the ankle joints and corresponding DRG. Similarly, it has been reported that methotrexate treatment reduces the severity of joint inflammation and destruction, partly due to its inhibitory effect on sensory neuropeptides (61).

Chronic pain is a major feature of inflammatory joint diseases and is maintained in part by long-lasting neuroplastic changes in the spinal cord. Previous studies demonstrated that the ubiquitin proteasome system is one of the systems involved in the maintenance of chronic pain by regulating proteins at pre- and post-synaptic levels (*see also section 1.4.2*).

1.4 THE UBIQUITIN PROTEASOME SYSTEM (UPS)

The degradation and processing of cellular proteins is critical for cell survival, growth, and cell division. Proteolysis via the proteasome pathway plays important roles in a variety of basic cellular processes. These processes are regulation of cell cycle and division, modulation of the immune and inflammatory responses, intracellular signaling and development and differentiation (62).

Cellular proteins are mainly degraded in two ways: lysosomal degradation and the ubiquitin proteasome system (UPS). Proteolysis in lysosomes is a non-specific process. In higher eukaryotes, membrane-associated and extracellular proteins captured during endocytosis (e.g. viral, bacterial) are destroyed in lysosomes. Degradation of the vast majority (80-90%) of intracellular proteins is via the UPS (63). The UPS degrades short-lived proteins under normal metabolic conditions, regulatory proteins or bulk degradation of long-lived proteins in the cytosol, nucleus as well as membrane and luminal endoplasmic reticulum in eukaryotic cells (62, 64).

Ubiquitin-mediated degradation of a protein involves two discrete and successive steps:

[1] Conjugation of multiple moieties of ubiquitin (Ub) to the protein substrate. Multiple copies of ubiquitin, a protein of 76 amino acids, bind covalently to available lysine residues on target proteins in a three-step process (Figure 3). In the first step Ub is activated by the action of E1 (Ub activating enzyme) and E2 (Ub conjugating enzyme). The substrate protein is then selectively targeted by the E3 (ubiquitin ligase) which ubiquitinates the protein. Further Ub molecules are added on to create a polyubiquitin chain.

[2] Recognition of polyubiquitinated proteins are by the 19S proteasome complex. Ub chain is cleaved by deubiquitinated enzymes (DUB), substrate protein is unfolded and enter into the 20S core for degradation. The substrate proteins are cleaved into smaller peptide chains (5-20 amino acids), which are further degraded into constituent amino acids and are recycled by the cell (62). The polyubiquitin chain is also broken down by the hydrolase enzymes and free Ub molecules are recycled by the cell (Figure 3) (65).

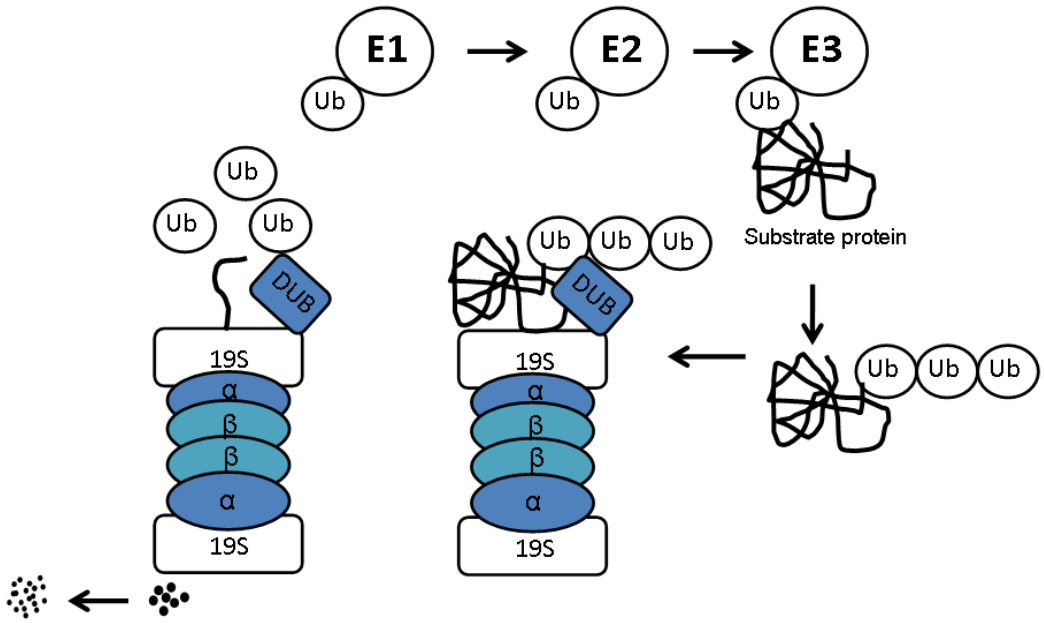

Figure 3. The ubiquitin-dependent degradation of protein

This process has been named the "ubiquitin-dependent degradation of protein" and was first discovered by A. Ciechanover, A. Hershko, and I. Rose who were later awarded the Nobel Prize in 2004 (66).

1.4.1 Proteasome Structure

The proteasome is a cylindrical shaped structure with a molecular weight of 1,500 to 2,000 kD, consists of two 19S regulatory complex and a core 20S catalytic complex (Figure 4). Hence it is also denoted the 26S proteasome (67, 68).

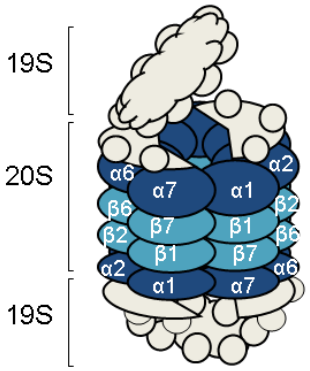

Figure 4. The 26S proteasome

1.4.1.1 The 19S proteasome subunit

Ubiquitin-tagged proteins are recognized by the 19S regulatory complex, where the ubiquitin tags are removed (Figure 4). ATPases with chaperone-like activity at the base of the 19S regulatory complex then unfold the protein substrates and feed them into the inner catalytic compartments of the 20S proteasome cylinder (69, 70). The opening into the 20S catalytic chamber is small (approximately 1.3 nm), and significant unfolding of the substrate is required for successful entering into the 20S subunit (71). A molecular gate (N-terminal tail of the α3-subunit) also guards the opening, but it is constitutively open when the 19S regulatory units are bound to the 20S proteasome (72).

1.4.1.2 The 20S proteasome subunit

It consists of two outer and two inner rings that are stacked to form a cylindrical structure with three compartments (68, 73). Each outer ring has seven alpha-subunits (α1 to α7), whereas each inner ring contains seven beta-subunits (ß1 to ß7). The 20S proteasome complex has chymotryptic, tryptic, and peptidylglutamyl-like activities (63, 67). It is conformationally flexible with active catalytic sites located on the inner surface of the cylinder where protein substrates bind. Proteins unfolded and without Ub tag, enter the inner chamber and are hydrolyzed by six active proteolytic sites on the ß-subunits (two sites each on the ß1-, ß2-, and ß5-subunits) into small polypeptides ranging from three to 22 amino acids in length. Proteins cannot enter the inner cylinder through the outer walls of the 20S proteasome because the gaps between the rings are tight (Figure 5) (71, 73, 74).

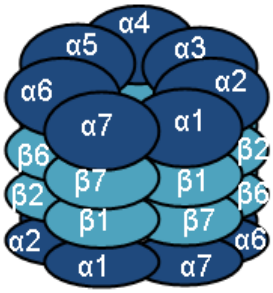

Figure 5. The 20S proteasome

1.4.2 The UPS and the CNS

In the CNS, the 26S proteasome is present in neurons, glia and synapses and regulates neuronal signalling, synapse formation and function (75). UPS regulates synaptic functions by controlling levels of pre-synaptic proteins (76). At the post-synaptic levels the UPS regulates the surface expression and internalization of NMDA- and AMPA-glutamate receptors (77).

1.4.3 The UPS and NF-κB activation

The UPS plays an essential role in the activation of transcription factor-κB (NF-κB) through proteolytic degradation of inhibitory protein IκB. NF-κB is a heterodimer composed of p50 and p65 subunits, and in non-stimulated cells NF-κB is retained in the cytoplasm in a latent form associated with inhibitory protein IκB. Following exposure of the cell to a variety of extracellular stimuli such as cytokines, viral and bacterial

products and stress, IκB is rapidly degraded by the 26S proteasome and the active heterodimer is translocated into the nucleus where it activates the transcription of corresponding genes (Figure 6) (78). NF-κB promotes transcription of genes which

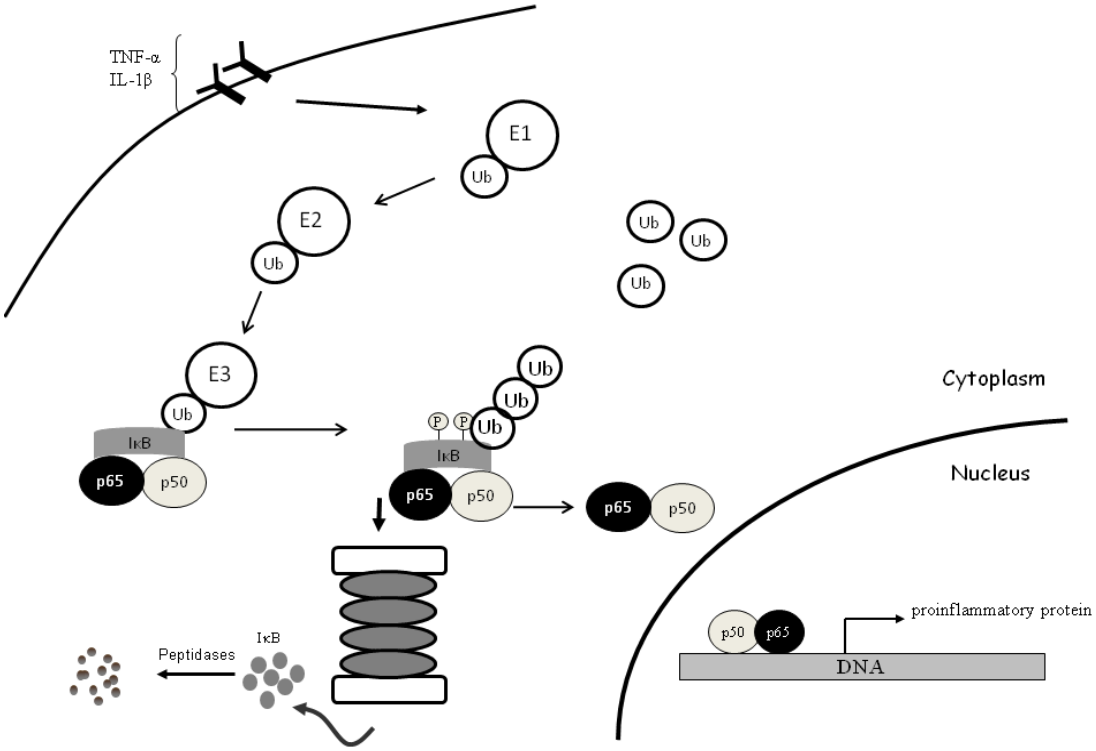

Figure 6. The activation pathway of NF-κB.

encode cytokines (TNF-α, IL-6, IL-1), stress response factors (Cyclooxygenase-2, NO), cell cycle regulators and anti-apoptotic proteins (IAP-1, Bcl-2 family) (79). It is believed that the pathological activation of NF-κB is a cause of many inflammatory diseases and has been an important target for therapeutic drug research in recent years (80).

1.4.4 The UPS and Diseases

With the broad spectrum of protein substrates and the complex enzymatic machinery involved in targeting them and practically all intracellular processes including cell cycle control, transcription, translation regulation, cell response to stress being controlled by the UPS, it is not surprising that the proteasome pathway is involved in the pathogenesis of malignant, autoimmune, and neurodegenerative diseases.

1.5 PROTEASOME INHIBITION

Proteasome inhibitors have provided a valuable research tool for studying the role of proteasome in cellular function and disease. Proteasome inhibition has considered as a

potential remedy for cancer, inflammation-related disorders and neurodegenerative diseases.

The UPS play a significant role in the degradation of regulatory proteins required for cell cycle progression and mitosis (62). A disruption in the regulation of these cell cycle proteins results in abnormal cell division and tumorogenesis (81). Proteasome inhibitors can cause cellular apoptosis in proliferating cancer cells by affecting various short-lived proteins, resulting in inhibition of NF-κB activity, increased activity of p53 and Bax proteins, and accumulation of cyclin- dependent kinase inhibitors p27 and p21 (17, 18). Preclinical studies show that malignant, transformed, and proliferating cells are more susceptible to proteasome inhibition than cells in a resting state (82, 83).

Bortezomib is the first inhibitor of the ubiquitin-proteasome pathway to enter clinical studies (84, 85). On the basis of a large, multicenter phase II clinical trial in which approximately one third of patients with advanced multiple myeloma (MM) had a significant response to therapy with bortezomib, on May 13th 2003, the US Food and Drug Administration granted approval for use of this drug in the treatment of patients with MM (86). The promising preclinical and clinical activity exhibited by bortezomib in MM and non-Hodgkin lymphomas (NHL) has confirmed the proteasome as a relevant and important target in the treatment of cancer. Several proteasome inhibitors are tested and are in pre-clinical and clinical phase (Table 4).

Table 4. List of proteasome inhibitors currently in pre-clinical and clinical testing (81).

Reagent	Clinical trials/status
Bortezomib (Velcade, PS341)	FDA approved for relapsed and refractory MM. Phase II trials in non-Hodgkin lymphomas (NHLs).
MLN519(PS-519)	Phase I trial completed. Intended for acute stroke and myocardial infarcation.
Epoxomicin	Pre-clinical
Ritonavir	Pre-clinical Approved for HIV treatment Phase II studies in tumour patients to be started

1.5.1 Toxicity

The clinical application of proteasome inhibitors might be limited due to potential side-effects of available compounds following chronic administration. Toxic affects might result from the accumulation of ubiquitinated proteins after inhibition of the 26S proteasome. The proteasome inhibitor bortezomib induced mild-to-moderate neurotoxic effects in rats (88) and peripheral sensory neuropathy in cancer patients that were given this compound chronically (89). The features of bortezomib neuropathy are characteristic for a small fiber neuropathy and are characterized by a more sensory than motor neuropathy. Thus there is a need for a non-toxic proteasome inhibitor.

1.6 UPS AND INFLAMMATORY JOINT DISORDERS

As discussed earlier (section *1.4.3*) UPS regulates NF-κB activation, which in turn promotes the expression of a variety of target genes involved in apoptosis and inflammation. In particular, an increase in the activity of NF-κB has been associated with the up-regulation of pro-inflammatory genes involved in several inflammatory conditions (78, 90).

The activation of NF-κB has been studied in several animal models of arthritis (91) and in the synovium of RA patients (92). Furthermore, the activation of NF-κB is implicated in the pathogenesis of RA (93, 94). An increased level of proteasome is present in serum in patients with different autoimmune diseases and is considered as a clinical marker of disease development (95, 96).

Elevated levels of MMP-1 (collagenase-1) in the synovial fluid and serum of RA patients (97, 98) and MMP-3 (stromelysin) in the synovial fluid from patients with both RA and OA have been determined (99). MMPs, especially MMP-3, are involved in the pathogenesis of OA through its ability to degrade proteoglycans (99, 100). Interestingly, it has been reported that NF-κB regulates synthesis of MMPs (101).

Progressive destruction of bone and articular cartilage plays a pivotal role in the pathogenesis of RA and OA. Previous studies have revealed that osteoclasts are responsible for bone destruction. NF-κB is essential for osteoclast formation and survival through the nuclear factor κB ligand (RANKL) pathway (102, 103). Abnormal activation of NF-κB signalling in osteoclasts has been observed in osteolytic conditions, including arthritis, Paget's disease of bone, and periodontitis (104). Inhibition or deletion of RANKL prevents bone destruction (103, 105).

1.6.1 Proteasome inhibition and inflammatory joint disorders

Proteasome inhibitors exhibit anti-inflammatory and anti-proliferative effects. Their use in diseases characterized by these processes is thought to be promising but still the effects of proteasome inhibitors on the pathogenesis of inflammatory join diseases is quite limited.

Proteasome inhibitors mainly regulate inflammation by inhibiting NF-κB activation. The inhibitors fulfill their task via a dual action: they sterically hinder the nuclear localization of NF-κB, thus retaining them in the cytosol in an inactive form bound to the inhibitory protein IκB, and block UPS-mediated processing of the p105 precursor to mature p50, which is a subunit of mature NF-κB. It has been observed that the inhibition of IκB reduces the severity of experimental arthritis (106).

The chondroprotective role of the proteasome inhibitor MG132 has been reported in one study *in vitro* and in a rat model of OA (107). The proteasome inhibitor bortezomib has been demonstrated to suppress human osteoclast formation and to promote maturation of osteoblasts (108, 109). Moreover, the proteasome inhibitor MG132 attenuates TNF-α-induced expression of MMP-3 in chondrocytes. However, the role of proteasome inhibitors in regulating RA and OA progression as well as modulating the clinical symptom, especially pain, is still unknown.

2 HYPOTHESIS AND AIMS OF THE THESIS

Our hypothesis is that transcription factor NF-κB critically regulates pain and inflammation in the inflammatory joint disorders. The neurogenic component of this regulation involves the expression of pro-nociceptive dynorphins and sensory neuropeptides.

With aim to test this hypothesis and to expand the knowledge about pain and inflammatory mechanisms in RA and OA the present study was designed.

More specifically, the aims of the study were:

1. To investigate the occurrence, localization and distribution of mediators of pain and inflammation in peripheral tissues and in the peripheral and central nervous system (Paper 1-4).

2. To assess a relationship between mediators of pain and inflammation with nociception and arthritis severity in RA and OA (Paper 2-4).

3. To explore the role of the transcription factor NF-κB in regulation of pain in bone and joint tissues, in addition to its regulation of inflammation. (Paper 2-3).

4. To analyze the effects of a reversible proteasome inhibitor, MG132, on pain and inflammation in RA and OA (Paper 2-4).

3 MATERIALS AND METHODS

3.1 ANIMAL MODELS OF ARTHRITIS

Experimental animal models have contributed tremendously to recent advances in the understanding of immunopathology of arthritis. In addition to provide basic insight into arthritis, animal models continue to provide the means to test novel experimental approaches for treatment. In this thesis, the potential ameliorative effects of the proteasome inhibitor MG132 (*N*-carbobenzoxyl-Leu-Leu-leucinal) on RA and OA are studied in two different rat models; adjuvant-induced arthritis and monosodium iodoacetate-induced osteoarthritis.

3.1.1 Adjuvant-induced Arthritis

To study pathogenesis of disease and to evaluate potential anti-arthritic drugs for clinical use several experimental models of arthritis have been developed in rats, mice, rabbits and monkeys. Most commonly used animal models include rat adjuvant arthritis (AIA), rat type II collagen arthritis (CIA), mouse type II collagen arthritis (CIA) and antigen induced arthritis in several species. Few spontaneously developing arthritis models such as DBA/1 male mice as well as some transgenic mouse models are also used (110).

In order to study the effect of MG132, the adjuvant-induced arthritis (AIA) rat model was used, which is a well accepted and most frequently used rat model of rheumatoid arthritis (RA) since early sixties (111, 112). In RA, there is a strong association of the immune system and the pathophysiology of disease. A similar association between the immune response induced by the complete Freund´s adjuvant and disease occurs in AIA. A single subcutaneous injection of a suspension of heat-killed *Mycobacterium butyricum* in paraffin oil into the base of the tail produces intense inflammation in joints, with accumulation of neutrophils, monocytes, macrophages and T cells in the synovium and synovial fluid (113-115). The inflamed, expanding synovium can develop into a pannus tissue, which attaches to and erodes cartilage and bone due to the release of proteases, hydrolases and cytokines from the leukocytes and synoviocytes (115-117). The pain, synovial hyperplasia, inflammation and destruction of cartilage and bone and even neurobiological changes in AIA resemble those that have been described for RA (118). Further, the AIA constitutes the only laboratory animal model of chronic pain that has been validated to a significant extent (119) and the AIA is one of the best-characterized experimental models in which to study the activity of anti-arthritic agents.

3.1.2 Monosodium iodoacetate-induced Osteoarthritis

Animal models of OA are used to study the pathogenesis of cartilage degeneration and to evaluate potential anti-arthritic drugs for clinical use. These include naturally occurring OA in knee joints of guinea pigs, mice, cows and horses. Surgically induced OA include medial meniscal tear in guinea pigs, rats, rabbits and dogs. Chemically induced cartilage degeneration by intra-articular iodoacetate injection and transgenic or knockout mouse models with defects in the expression of transcription factors, MMPs, angiogenic factors, or ECM proteins have provided insight into the mechanisms that control cartilage development (120-122).

The monosodium iodoacetate (MIA) model used in the present work is considered as a reliable and consistent experimental OA model with cartilage degeneration and pain that mimics pathological changes in OA patients. The MIA-induced OA is one of the best-characterized experimental models to study effects of drugs on the pathology of osteoarthritis (123). The injection of the metabolic inhibitor MIA into a knee joint of rats inhibits glyceraldehyde-3-phosphate dehydrogenase activity in chondrocytes, resulting in disruption of glycolysis and eventually cell death. The progressive loss of chondrocytes results in histological and morphological changes of the articular cartilage and a pain related behavior, closely resembling those seen in osteoarthritis patients.

The initial period of knee swelling in MIA-induced OA implies a transient synovial inflammation, which may have clinical correlation with early synovial inflammation that predicts development of OA in the knee (124). It is then resolved for the remaining duration of the study, whereas in the human disease, recurrent inflammatory phases are noted. Free nerve endings have been identified in rat (125) and human (126) synovium and probably play an important role in joint pain sensation.

3.2 CLINICAL EVALUATION OF EXPERIMENTAL ARTHRITIS

3.2.1 Inflammation

Disease progress can be graded in different ways. Most common is to evaluate the number of affected joints in each paw. This correlates with the evaluation system used for human arthritis patients and grade both redness and swelling. Other methods of evaluation are also possible, such as the measurement of swelling over the ankle or tarsus joints or measurement of total paw swelling.

The severity of arthritis was evaluated by a scoring system in the adjuvant arthritis model (Paper 2). The basis for the system is a clinical evaluation of the affected paws. The severity of inflammation in each paw was graded (1-3) by two observers without knowledge of the treatment for redness and paw swelling. The total score was the cumulative value for four paws, with a maximum of 12 for each rat. Rats were considered to be arthritic if the paw score was above 2.

In OA model (Paper 4) inflammation was evaluated by measuring the swelling of right and left knee joints recorded at the anterior posterior position using a digital calliper (Limit, UK). Joint swelling was measured in mm and was determined as the ratio between the left and right knee joints.

3.2.2 Pain

Assessing the level of pain in animals is a key element in pain research. Current pain assessment techniques are mainly limited to estimate animal responses elicited by stimuli to the, presumably, affected area using thermal, tactile and mechanical stimuli. These responses are invariably reflexive in nature, and are informative only about the thresholds of the sensory variables being probed; thermal, tactile, or mechanical. These thresholds are obviously important in the assessment of painful states, but are not the complete perceptual pain experience from a whole organism perspective.

The hind paw withdrawal threshold (PWT) to mechanical pressure was assessed with an algesimeter (Ugo Basile, Italy). A wedge-shaped pusher was applied to the dorsal surface of the hind paw at a steadily increasing pressure. The PWT was determined

when the animal removed the foot from the apparatus, and the pressure, in grams, necessary to evoke paw withdrawal was recorded. A cutoff threshold of 220 g was preset to prevent tissue damage. Before measuring the baseline values, the rats were habituated to the algesimeter. All measurements were made by two observers without knowledge of the treatment regimen (Paper 2, 4).

3.2.3 Bone and cartilage destruction

Destruction of bone and cartilage was assessed in AIA and in MIA-induced OA by radiology and histological analysis using a semi-quantitative grading scoring system.

3.2.3.1 Radiology

Radiographs of ankle (AIA) and knee joints (OA) of anesthetized rats were taken with the help of a dental X-ray machine (Heliodent DS, Siemens AG, Bensheim, Germany). X-ray films were developed and scanned with a HP ScanJet II scanner (Hewlett-Packard, Singapore) and saved into a computer. Lateral view radiographs of ankle joints (Paper 2) were evaluated by two observers without knowledge of treatment for osteoporosis, cartilage loss and for bone erosion using a subjective 0-3 grading scale where 0 = normal, 1 = mildly, 2 = moderately, and 3 = severely affected joint.

Knee radiographs (Paper 4) were taken from the anterior-posterior and lateral views of joints and analysed for thinning of joint space, severity of subchondral bone sclerosis and osteophyte formation using a scale 0-3.

3.2.3.2 Histology

Ankle joint. Bilateral hind ankle joints (AIA) were stained with haematoxylin and eosin to assess the pathological changes in the ankle joints and to monitor the effects of MG132 treatment (Paper 2). Pathological changes in the ankle joint were scored (0-3) by two observers without knowledge of treatment and were assessed according to the cartilage destruction and bone erosion, and inflammation of the synovium.

Knee Joint. Knee joints (MIA) were stained with toulidine blue and hematoxylin and eosin (Paper 4). Histological evaluation was performed by two observers without knowledge of treatment on the weight-bearing area of the medial femoral condyle and graded on a scale of 0-14, using the Mankin scoring system. Mankin scoring system, which was developed for the assessment of human articular cartilage, has also been used for grading of animal cartilage. This system assesses changes in structure (0-6 points), cellular abnormalities (0-3 points), matrix staining (0-4 points) and tidemark integrity (0-1), with a maximum of 18 points per section (127, 128).

Pathological changes in the synovium from knee joint was scored and assessed according to the synovial lining layer and the subsynovial tissue with a maximum of 18 points per section. The synovial lining layer was scored according to; hyperplasia of synovial lining cells (0-3 points), hypertrophy of synovial lining layer (0-3 points), and infiltration of inflammatory cells (0-3 points); and the subsynovial tissue were analyzed as: proliferation of granulation tissue (0-3 points), vascularization (0-3 points), and infiltration of inflammatory cells (0-3 points) (128).

3.3 BIOCHEMICAL TECHNIQUES USED IN THIS THESIS

Detailed description of radioimmunoassay (Paper 1), in Situ hybridization (Paper 1), Western Blotting (Paper 1), electro mobility shift assay (Papers 2, 3), quantitative RT-

PCR (Papers 3, 4) and immunohistochemistry (Papers 1-4) can be found in the individual papers.

4 RESULTS AND DISCUSSION

4.1 DISTRIBUTION OF PRODYNORPHIN IN THE RAT CNS

Paper 1

Up-regulation of PDYN mRNA and mature dynorphins (DYNs) has been detected in animal experiments under conditions of neuropathic, cancer and inflammatory pain (129, 130) suggesting the pro-nociceptive role of these peptides. In these and other previous studies, the DYN system has been studied at the levels of mRNA and mature peptides but not at the protein precursor levels. In our first study we analyzed cellular localization and distribution of PDYN, the protein precursor to dynorphins in rat brain, and regulation of PDYN cleavage to mature proteins in response to external signals. We assume that this approach would characterize regulation of the PDYN biogenesis that is relevant for regulation of pain transmission and perception.

The PDYN distribution pattern in the rat brain as determined by IHC and western blotting was similar to that of DYN peptides. This distribution was generally consistent with previous analyses of DYNs with the highest levels being observed in the amygdala, hippocampus and stratum, with lower amounts in the cerebral cortex (131, 132).

PDYN in an unprocessed form was also detected in brain structures such as the ventral trigeminal area (VTA) and the hypocampal CA3 regions that do not have cell bodies of neurons producing PDYN but that contain axons and axon terminals originating from PDYN-ergic neurons in the ventral strianum and dentate gyrus, respectively. Using western blotting it was revealed that PDYN was present in all four regions (dentate gyrus, CA3 region of the hippocampus, nucleus accumbens and VTA) and that its levels in the VTA and CA3 regions of the hypocampus were slightly lower than those in the ventral straitum and dentate gyrus, respectively (Figure 7A, B).

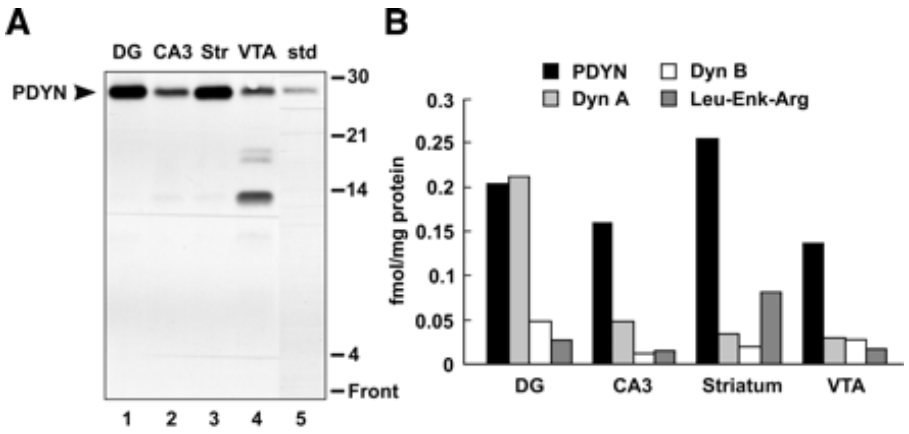

Figure 7. Molar content of PDYN and mature neuropeptides (Dyn A, Dyn B, and Leu-enkephalin-Arg) in rat dentate gyrus and striatum where somata of PDYN-containing neurons are located and in CA3 subfield of hippocampus and VTA that both contain only projections of PDYN-containing neurons by *A)* WB and *B)* RIA.

In situ hybridization detected negligible levels of PDYN mRNA in the CA3 and VTA regions. Taken together, our results confirm that the precursor was not locally

synthesized in these regions and PDYN is transported to and stored in axon terminals. To our knowledge, this is the first study indicating non-classical role of PDYN. Further, it was observed that PDYN form insoluble oligomers stabilized by disulphide bonds that possibly represent the storage form of the protein in secretary granules in the axon terminals and dendrites.

The best scenario would have been to analyse PDYN in the brain during inflammatory conditions. The spinal cord reflects an extension of the CNS and has traditionally been used to analyze pro-nociceptive mediators in inflammatory conditions (130). The occurrence of DYNs in some C-fibers and co-localization of DYNs with SP and CGRP in the spinal dorsal horn has been previously indicated (133). In addition, it has been demonstrated that DYN up-regulation is a necessary event in the maintenance of neuropathic pain (134). The expression of endogenous opioid peptides and their receptors in bone and joint tissues and their co-existence with sensory neuropeptides in peripheral nerves (135, 136) signifies their role in inflammatory joint diseases. DYNs may be regulated at the transcriptional level, due to stimulation of the processing of the precursor molecule, or inhibition of degradation of these peptides. In this study we addressed the possibility of regulation of DYN at the post-translational level.

At axon terminals PDYN may be processed to mature peptides prior to their release from secretary granules or secreted as intact precursors. To test this, MIN6 cells were stimulated and total levels of the mature peptide in cells and medium were elevated in the presence of a high concentration of K^+. The K^+ -evoked depolarization of PDYN producing cells increases the total amount of DYNs in cells and medium demonstrating that PDYN processing is activated. Depolarization of neuronal cells stimulated processing of PDYN into DYNs and Leu-enkephalin-Arg. Stimulation of PDYN processing in axon terminals and dendrites by neuronal activity and extracellular signals may represent a mechanism for the local regulation of synaptic transmission. This mechanism may be relevant for several neuropathological conditions, including neuropathic and inflammatory pain.

One of the systems actively involved in synaptic transmission and processing and stimulated secretion of synaptic proteins including DYNA and CGRP release and postsynaptic actions of DYNA is the UPS (137).

4.2 ROLE OF UPS ON PAIN AND INFLAMMATION IN ADJUVANT-INDUCED ARTHRITIS

Papers 2 and 3

In the second and third study we investigated the role of the UPS in chronic joint inflammation and inflammatory pain by systemically inhibiting this system in adjuvant arthritis. UPS plays an essential role in the activation of transcription factor NF-κB which is involved in the expression of pro-inflammatory mediators such as cytokines and cell adhesion molecules (138). In addition, UPS regulates synaptic functions by controlling levels of pre- and post-synaptic proteins under normal conditions and is required for the maintenance of neuropathic and pathological pain induced by sustained morphine exposure (87, 139). However, the information about the role of UPS in the pathogenesis of chronic arthritic conditions is scarce.

The activation of NF-κB, in arthritic ankle joints, was investigated by IHC and EMSA. Our results indicate an increase in p50 (a subunit of NF-κB) positive cells in the synovium and in cartilage during arthritis. Our results are in accordance with previous observations indicating an increase in the p50 and p65 positive cells in experimental model of arthritic (91), and in synovium from RA patients (93, 94). Moreover, we observed an increased DNA-binding activity of NF-κB and p50 in arthritic joints. Repeated subcutaneous administration of reversible proteasome inhibitor MG132 at the onset of arthritis, significantly down-regulated the severity of arthritis as indicated by reduced arthritis index (Figure 8A) and the p50 expression and DNA binding activity of both NF-κB and p50 homodimers in arthritic rats. Our results verify that activation of NF-κB contributes to the pathogenesis of arthritis and an early intervention could give promising results. Further, our results confirm that the processing of the p100 precursors into mature p50 subunits is regulated by the UPS.

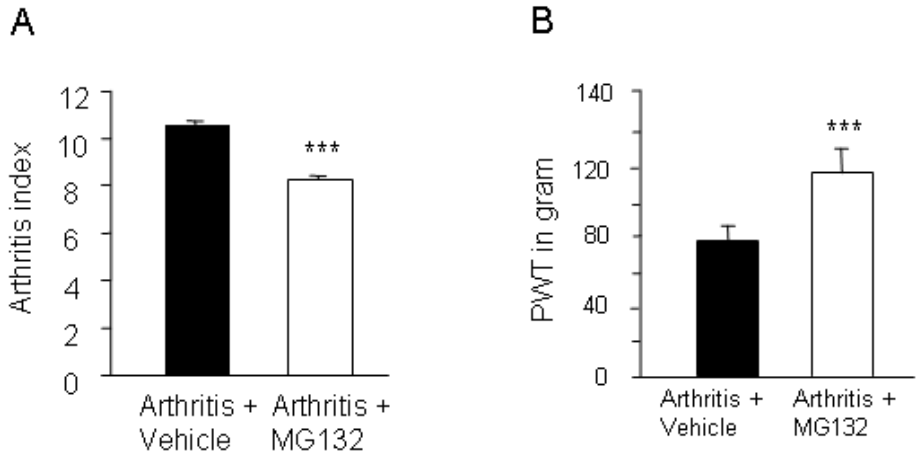

Figure 8. Effects of proteasome inhibitor MG132 on (A), arthritis index and (B), on the hind paw withdrawal threshold (PWT) on day 28.

In order to study UPS effects on pain, SP and CGRP expression was studied in the periosteum and synovium of arthritic ankle joints; these are structures that are pain-sensitive and prone to inflammation. The increased SP and CGRP expression coincided with decreased pain thresholds as measured by paw withdrawal threshold (PWT) in arthritic rats (Figure 8B). It has been implicated that joints that developed severe arthritis had a higher density of innervations by SP-containing sensory neurons, and a higher SP content, than joints that developed mild arthritis (140). Neural depletion of SP by capsaicin eliminates paw swelling and tenderness within the inflamed joint (141). Correspondingly, MG132 increased PWT in arthritis rats and levels of SP and CGRP were down-regulated in arthritic joints indicating that UPS regulates inflammation induced pain behaviour. Thus, our results show for the first time that UPS-mediated protein degradation likely is involved in peripheral sensitization. In addition they verify that SP and CGRP expression is UPS mediated. Interestingly, it has been observed that proteasome inhibitors MG132 and epoxomicin can prevent the development of behavioural signs of neuropathic pain and abolish abnormal pain induced by sustained morphine exposure (87, 142) These compounds inhibited the release of DYNA and CGRP and normalized molecular changes in the spinal cord contributing to central sensitization (87, 142).

Our radiographic and histological analysis revealed that the subchondral bone resorption which is a characteristic feature of adjuvant arthritis was mitigated by MG132. MG132 possibly prevented bone resorption by interfering osteoclast activation through the RANKL signalling pathway that is under control of NF-κB (102). This assumption is supported by recent *in vitro* and *in vivo* studies indicating that the proteasome inhibitor bortezomib directly suppressed human osteoclast formation and promoted maturation of osteoblasts (108, 109). Thus, our results suggest that targeting of UPS by MG132 reduces pain and inflammation and bone destruction in adjuvant arthritis.

Paper 3

In this study we further explored the role of UPS on molecular changes in the DRG and in the spinal cord, by systemically inhibiting this system in adjuvant arthritis. The DRG and the spinal cord actively participate in the peripheral and central sensitization.

It has been previously demonstrated that proteasome inhibitors MG132 and epoxomicin can both prevent the development of behavioural signs of neuropathic pain and abolish abnormal pain induced by sustained morphine exposure (134, 135). These compounds also inhibited the enhanced DYN and CGRP release and reduced central sensitization by normalizing molecular changes in dorsal horn neurons (137). Inflammatory and neuropathic pain activates similar central neuronal mechanisms and neurotransmitters from the primary afferents including SP and CGRP. We aimed that proteasome inhibitors used to treat experimental neuropathic pain may also be successful for the treatment of inflammatory pain.

PCR and IHC analyses revealed a significant increase in the synthesis and expression of SP in the DRG and in the spinal cord in arthritic rats. In the spinal cord, SP positive nerve fibers were identified in the dorsal horn of the spinal cord, predominantly in lamellae I, II, and a few in the deep layers. Up-regulated SP expression correlates with arthritis severity and nociceptive behavior of arthritic rats. This concord with previous observations that the altered expression of SP and CGRP is critical for the modulation of pain and inflammation (141, 143).

Interestingly, no change in PDYN expression was observed in the DRG neurons of arthritic rats by PCR or IHC. In contrast, strong up-regulation in PDYN gene expression was observed in the spinal cord. IHC analysis revealed PDYN immunoreactivity in lamellae I, II and V. Our results are in agreement with previous observations indicating that peripheral inflammation induces a dramatic up-regulation of PDYN biosynthesis in nociceptive neurons of the spinal dorsal horn (133, 144). MG132 treatment reversed the up-regulated expression of SP both in the DRG and in the spinal cord of arthritic rats and PDYN expression in the spinal cord.

Our data suggest that MG132 exert its anti-inflammatory and anti-nociceptive responses by the attenuating SP expression in DRG neurons and SP and PDYN expression in the spinal dorsal horn. Our findings indicate that effects of proteasome inhibitor MG132 on chronic inflammatory pain are mediated through several protein systems, including those controlling dynorphin and sensory neuropeptides SP and CGRP release. Thus, by the use of an inhibitor of the UPS we propose that the UPS acts directly in the DRG in the spinal cord, in addition to its effect in the joints.

4.3 EFFECTS OF UPS INHIBITION ON OSTEOARTHRITIS PAIN AND DISEASE PROGRESSION

Paper 4

In this study we investigated the effects of MG132 on OA pain and disease progression in the MIA-induced OA model. To our knowledge, this is the first report of the effects of UPS inhibition on OA pain. Mechanisms of pain in OA are still unclear but probably contributed by sensitization of nociceptors in the synovium and subchondral bone. The aim was to study the role of UPS in OA pain and on disease progression.

In OA rats, radiographic and histological analysis revealed that pathological changes in knee joints, loss of proteoglycan and thinning of the articular cartilage were similar to human OA. Biochemical analysis indicated an up-regulation in the expression of matrix metalloptoteinase-3 (MMP-3) in the cartilage, MMPs has ability to degrade proteoglycans, and may be involved in the pathogenesis of OA (100, 101). An increased expression of SP and CGRP in inflamed knee joints was observed, especially in the synovium and in the subchondral bone, and coincided with decreased pain thresholds in OA rats. Administration of MG132 reversed pain behaviour and the expression of MMP-3 altered during OA.

Our observations, indicating a chondroprotective role of MG132 in OA, concord with previous *in vitro* and *in vivo* studies (107) and further explore that the chondroprotective role of MG132 probably is mediated through decreased MMP-3 expression in OA cartilage. Interestingly, MMP-3 synthesis is regulated by the NF-κB, the activation of which is under the control of UPS. However, it has been reported earlier that proteasome inhibitors, bortizomib and MG262 can induce apoptosis in proliferating chondrocytes in the growth plate of young mice (145) emphasizing the need of further studies to scrutinize the clinical use of proteasome inhibitors.

The progressive loss of articular cartilage results in subchondral bone exposure. The subchondral regions of long bones are highly vascularised (146). The subchondral ischemia and increased venous pressure due to subchondral bone exposure as well as joint loading may induce the liberation of non-adrenergic/non-cholinergic pain mediators such as SP and CGRP in the subjacent bone inducing pain (147). Clinical evidence demonstrates that SP and CGRP levels were increased in the capsule of hip joints and soft tissues in patients with painful OA (148). MG132 abolished the upregulated expression of SP and CGRP especially in subchondral bone in the knee joints.

A robust effect with reversal of pain behavior, attenuated cartilage destruction and decreased SP, CGRP and MMP-3 expression was observed with MG132 administration. Thus, these results suggest that the pain and cartilage destruction in OA are regulated by the UPS. These findings may be relevant for human OA, paving the way to develop a novel pharmacotherapy regulating pain as well as the disease progression.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In my thesis work I provide evidence that PDYN in the brain is transported to and stored in axon terminals in the CA3 regions of the hypocampus and in the VTA in an unprocessed form. PDYN form insoluble oligomers which are stabilized by disulphide bonds and possibly represent the storage form of the protein in the axon terminals and dendrites. Depolarization of neuronal cells stimulated processing of PDYN into mature DYNs and their release. Activation of PDYN processing possibly represents a novel mechanism for the local regulation of synaptic transmission in the CNS.

Furthermore, I provide evidence that pain and inflammation in animal models of arthritis is regulated by UPS. The severity of arthritis and inflammatory pain is reduced by using a synthetic small molecule MG132, which has an inhibitory effect on UPS. Moreover, the treatment of arthritic rats by MG132 reversed the up-regulated expression of SP and CGRP in the affected joints and SP and PDYN in the DRG and in the spinal cord. The disease modifying effects of MG132 was associated with reduced NF-κB activation at the site of inflammation as well as in the spinal cord. Probably, by selective control of NF-κB activity by proteasome inhibitors in the early stages of RA and OA, a better symptoms control could be achieved.

It is known that NF-κB is an important transcription factor involved in a wide range of clinical disorders including the inflammatory joint disorders RA and OA. It is important to investigate the effects of MG132 on human synovial samples. In preliminary studies of human RA synovial samples, we recorded a down-regulation of IL-6 production by 80% when MG132 was added to the biopsy culture. In contrast, IL-8 production was unaffected (Wähämaa and Ahmed, unpublished data, 2010). Future studies to evaluate the effect of MG132 on NF-κB activation and cytokine production as well as on the release of SP and CGRP in synovial tissues would provide promising results.

In this thesis work I evaluated anti-nociceptive and anti-inflammatory effects of MG132 during the early and late inflammatory phase of adjuvant and osteoarthritis. The results indicate the probable involvement of UPS both in the early inflammatory as well as in late neurogenic phase of pain. Furthermore, our results suggest that the beneficial effects of MG132 are mediated probably by regulating the expression of dynorphins, SP and CGRP in the DRG and spinal cord and their possible release in the peripheral tissues. These results are supported by previous studies indicating that proteasome inhibitors MG132 and epoxomicin decreased pain by attenuating DYNA and CGRP release in the spinal cord (137, 142). Recently, it has been shown that UPS regulates the surface expression and internalization of NMDA- and AMPA-glutamate receptors at post-synaptic levels (77). It has been implicated that DYNA-induced mechanical allodynia and hyperalgesia can be prevented with NMDA-receptor antagonists (149). Future investigations exploring UPS mediated glutamergic regulation would help to understand the mechanism behind chronic inflammatory pain.

Molecular changes responsible for pain sensitivity are not restricted to neurons in the dorsal horn, and there is growing evidence to suggest the involvement of glia- and of glia-neuronal signalling in initiating and sustaining enhancement of nociceptive transmission (150). In particular, a role has emerged for microglia in pain

hypersensitivity following nerve injury (150). Thus, by expanding the understanding of cellular and molecular signalling mechanisms in the dorsal horn, which includes both neurons and glia, new strategies targeting UPS will be beneficial to reverse chronic inflammatory pain conditions.

In the peripheral tissues selective blocking of nociceptive signals would be an attractive approach. Recently, expression of the two main classes of acid-sensing nociceptors, the acid-sensing ion channels (ASICs) and the transient receptor potential channel vanilloid subfamily member 1 (TRPV1), has been reported in the sensory neurons innervating bone (151). The acidic microenvironment created by bone-resorbing osteoclasts activates these acid-sensing nociceptors, causing bone pain. Maturation of osteoclasts is regulated by the UPS, thus emphasizing the need to target these receptors in peripheral tissues.

Present work revealed that MG132 attenuated bone and cartilage destruction in adjuvant arthritis and OA in rat. MG132 possibly prevents destructive changes by attenuating MMPs expression and by interfering osteoclast activation through the RANKL signalling pathway, both processes being under the control of NF-κB (102). This assumption is supported by recent *in vitro* and *in vivo* studies indicating that proteasome inhibitor bortezomib directly suppressed human osteoclast formation and promoted maturation of osteoblasts (108, 109). More direct evidence will be required to consider use of MG132 as a disease-modifying agent in both RA and OA.

Taking into account that UPS controls important functions in eukaryotic cell, proteasome inhibitors could have been considered as toxins without any therapeutic value. Unexpectedly, proteasome inhibitors are well-tolerated drugs and do not produce adverse effects in normal cells even at high doses. Though, clinical trials indicate that use of bortezomib induce peripheral sensory neuropathy in patients (152), that might limit its therapeutic use. However, the reversible proteasome inhibitors like MG132 used in present thesis work apparently did not produced any toxic effects and was well tolerated. The use of reversible proteasome inhibitors can therefore be considered as a better alternative. It will be a future challenge to develop drugs specifically targeting the UPS or more specifically UPS E3 that select proteins for the UPS-mediated degradation in order to treat inflammatory joint disorders.

In conclusion, our results suggest that UPS is one of the intracellular pathways that are critically involved in the development of joint inflammation and inflammatory pain. It is expected that novel and safe proteasome inhibitors with limited adverse effects would be available targeting joint changes, pain and inflammation in inflammatory joint disorders.

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