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Regulation of Inducible Nitric Oxide Synthase Expression and Nitric Oxide Production in Osteoarthritic Cartilage and Chondrocytes

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ACADEMIC DISSERTATION

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the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building B,
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Contents

List of original communications

This thesis is based on the following original communications, referred to in the text by their Roman numerals I-V.

I Vuolteenaho K, Moilanen T, Al-Saffar N, Knowles RG and Moilanen E (2001): Regulation of the nitric oxide production resulting from the glucocorticoid-insensitive expression of iNOS in human osteoarthritic cartilage. Osteoarthritis Cartilage 9:597-605.

II Vuolteenaho K, Moilanen T, Hämäläinen M and Moilanen E (2002): Effects of TNFα-antagonists on nitric oxide production in human cartilage. Osteoarthritis Cartilage 10:327-332.

III Vuolteenaho K, Moilanen T, Hämäläinen M and Moilanen E (2003): Regulation of nitric oxide production in osteoarthritic and rheumatoid cartilage: role of endogenous IL-1 inhibitors. Scand J Rheumatol 32:19-24.

IV Vuolteenaho K, Moilanen T, Jalonen U, Lahti A, Nieminen R, van Beuningen HM, van der Kraan PM and Moilanen E (2005): TGFβ inhibits IL-1 -induced iNOS expression and NO production in immortalized chondrocytes. Inflamm Res, in press.

V Vuolteenaho K, Kujala P, Moilanen T, Hämäläinen M and Moilanen E (2005): Aurothiomalate and hydroxychloroquine inhibit NO production in chondrocytes and in human OA cartilage. Scand J Rheumatol, in press.

Abbreviations

1400W	N-[3-(aminomethyl)benzyl]acetamidine, NOS inhibitor
AP-1	activator protein-1
BH_4	(6R)-tetrahydrobiopterin
$[Ca^{2+}]_i$	intracellular free calcium
CaM	calmodulin
cGMP	3', 5'-cyclic monophosphate, second messenger
DAHP	diaminohydroxypyrimidine, inhibitor of BH_4 synthesis
DMARDs	disease-modifying antirheumatic drugs
eNOS	endothelial nitric oxide synthase
Erk1/2	extracellular signal-regulated kinase 1 and 2
ICE	interleukin-1β converting enzyme or caspase-1
IFN	interferon
IκB	inhibitor κB
IKK	inhibitor κB kinase complex
IL	interleukin
IL-1R	interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
Jnk	c-Jun N-terminal kinase
KO	knockout, gene deletion
L-NAME	N-nitro-L-arginine methyl ester, NOS inhibitor
L-NIL	N-iminoethyl-L-lysine, NOS inhibitor
L-NIO	L-N-iminoethyl-L-ornithine, NOS inhibitor
L-NMMA	N-monomethyl-L-arginine, NOS inhibitor

LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
NF-κB	nuclear factor κB
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
OA	osteoarthritis
PDTC	pyrrolidine dithiocarbamate, NF-κB inhibitor
PD98059	2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one, Erk1/2 inhibitor
RA	rheumatoid arthritis
SB203580	4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazol, p38 inhibitor
sIL-1R	soluble interleukin-1 receptor
Smad	mediators of TGFβ family signals, merger of the Mad (mother against decapentaplegic) gene in Drosophilia and the related Sma genes in Caenorhabditis elegans to the vertebrate genes and their products (Derynck et al. 1996)
SP600125	anthra(1,9-cd)pyrazol-6(2H)-one, Jnk inhibitor
sTNFR	soluble tumor necrosis factor α receptor
TACE	tumor necrosis factor α converting enzyme
TGFβ	transforming growth factor β
TGFβR	transforming growth factor β receptor
TNFα	tumor necrosis factor α
TNFR	tumor necrosis factor α receptor

Abstract

Osteoarthritis (OA) is the most common cause of musculoskeletal disability and pain in the Western world. The characteristic feature of OA is destruction of the articular cartilage, although pathognomonic features are found throughout the entire joint. According to current viewpoint, OA is regarded as a local slowly progressing inflammatory condition, in which the chondrocyte plays an active role by releasing inflammatory and destructive mediators. The proinflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor α (TNFα), enhance the destructive processes in OA cartilage. Both of these cytokines are capable of inducing nitric oxide (NO) production in chondrocytes and NO mediates many of the destructive effects of IL-1 and TNFα in the cartilage. Inhibitors of NO synthesis have shown retardation of clinical and histological signs and symptoms in various models of arthritis as well as in experimental OA.

Current therapy of OA combines non-pharmacological and pharmacological treatment to relieve pain, maintain or improve joint function, and decrease disability. At the moment there is no treatment available that could prevent or retard the disease progression in OA. NO production in chondrocytes represents a promising target to inhibit or to depress the destructive processes going on in the cartilage. The aim of the present study was to investigate the signaling pathways in the induction of iNOS expression and NO production in OA cartilage and in chondrocyte culture activated by IL-1. In addition, the pharmacological regulation of NO production and the effects of currently available disease-modifying antirheumatic drugs (DMARDs) on NO production were investigated.

In the present study, OA cartilage was found to produce NO spontaneously via inducible nitric oxide synthase (iNOS). Protein tyrosine kinases and nuclear factor κB (NF-κB) activation is involved in the iNOS induction. The results indicated that OA cartilage actively regulates responses to cytokines by producing endogenous cytokine antagonists IL-1 receptor antagonist (IL-1Ra), soluble IL-1 receptor II (sIL-1RII) and soluble TNFα receptor I and II (sTNFRI and sTNFRII). A highly selective iNOS inhibitor 1400W further enhanced IL-1 -induced IL-1Ra production by OA cartilage suggesting the presence of a positive feedback loop between IL-1 and NO as IL-1 -induced NO production promotes cartilage destruction by IL-1 by suppressing production of protective IL-1Ra. In addition to cartilage destructive mediators i.e. IL-1 and TNFα, the concentrations of anabolic transforming growth factor β (TGFβ) in synovial fluid are increased in OA. TGFβ inhibited IL-1 -induced NO production and iNOS expression by enhancing iNOS protein degradation. This finding suggests additional mechanism for TGFβ to counteract the destructive effects of IL-1 in OA and points to the importance of post-transcriptional regulation in NO production together with transcriptional regulation.

Glucocorticoids are commonly used as intra-articular injections in the treatment of knee OA. Although glucocorticoids have been shown to suppress NO production in various cell types, IL-1 -induced NO production in OA cartilage and chondrocytes was insensitive to dexamethasone. From the disease-modifying antirheumatic drugs (DMARDs) studied, aurothiomalate and hydroxychloroquine suppressed IL-1 -induced NO production in chondrocyte cultures and in OA cartilage probably due to suppression of NF-κB activation. The TNFα-antagonists, etanercept and infliximab, suppressed TNFα -induced NO production in OA cartilage but had no effect on NO production induced by IL-1, LPS or IL-17. These data suggests that TNFα is not an autacoid mediator in these processes. In summary, aurothiomalate, hydroxychloroquine, etanercept and infliximab suppressed induced NO production in OA cartilage pointing to therapeutic potential for these drugs in the treatment of OA. The inhibitory effect also serves as a mechanism of action by which aurothiomalate and TNFα-antagonists can retard the progression of RA, because the inhibitory effects were achieved at drug concentrations found *in vivo* after administration of antirheumatic doses of the drugs.

Tiivistelmä

Artroosi l. nivelrikko (OA) on tavallisin nivelkipuja ja toiminnanrajoitusta aiheuttava sairaus ja merkittävä kansanterveysongelma. Artroottiselle nivelelle tyypillisiä muutoksia on kaikissa nivelrakenteissa, mutta nivelruston tuhoutuminen tulehduksellisen prosessin seurauksena on artroosin keskeisin piirre. Rustotuhon kannalta keskeiset sytokiinit interleukiini-1 (IL-1) ja tuumorinekroositekijä α (TNFα) ovat tehokkaita ruston typpioksidi (NO) -synteesin käynnistäjiä. *In vitro* -kokeissa typpioksidilla on todettu olevan useita rustoa tuhoavia ja niveltulehdusta voimistavia vaikutuksia. Typpioksidituottoa estävien lääkeaineiden vaikutusta on tutkittu kokeellisessa artriitissa ja artroosissa, joissa niillä oli selvä niveltuhoa hidastava vaikutus.

Tällä hetkellä artroosin hoito on lähinnä kivun hoitoa ja toimintakyvyn ylläpitoa lääkkeillä ja kuntoutuksella ja pidemmälle edenneissä tapauksissa tekonivelkirurgialla. Typpioksidi on keskeinen rustotuhoa edistävä välittäjäaine artroosirustossa ja on perusteltua esittää hypoteesi siitä, että typpioksidituottoa estävillä lääkeaineilla voitaisiin hidastaa artroosin aiheuttamien rustomuutosten etenemistä. Tämän tutkimuksen tavoitteena oli selvittää niitä solunsisäisiä mekanismeja, jotka johtavat typpioksidituoton käynnistymiseen rustosoluissa ja artroosirustossa sekä tutkia lääkekehityksen näkökulmasta niitä keinoja, joilla artroosiruston typpioksidisynteesiä voitaisiin säädellä.

Tutkimuksessa havaittiin, että artroosirusto tuottaa typpioksidia spontaanisti rustokudosviljelmässä johtuen indusoituvan typpioksidisyntaasi -entsyymin (iNOS) ilmentymisestä artroosirustossa. Tyrosiinikinaasien ja transkriptiotekijä NF-κB:n aktivoituminen ovat iNOS-entsyymin induktiossa keskeisiä vaiheita. Tutkimuksessa havaittiin ruston osallistuvan aktiivisesti typpioksidituoton säätelyyn tuottamalla endogeenisiä sytokiini-antagonisteja, kuten IL-1 reseptoriantagonistia (IL-1Ra), liukoista IL-1 II reseptoria (sIL-1RII) ja liukoista TNFα I ja II reseptoria (sTNFRI ja II). IL-1 sai aikaan IL-1Ra -tuoton artroosirustossa ja selektiivinen iNOS estäjä, 1400W edelleen voimisti tätä. Tulos viittaa IL-1:n ja typpioksidin toisiaan voimistavaan rustotuhoa edistävään vaikutukseen, koska IL-1 -indusoitu typpioksidituotto esti suojaavan IL-1Ra:n tuottoa. Artroosissa rustotuhoa edistävien IL-1:n ja TNFα:n ohella anabolisen kasvutekijän, transformoivan kasvutekijä β:n (TGFβ), tuotanto on kiihtynyt. Tulostemme mukaan TGFβ estää kondrosyyttien typpioksidituottoa aiheuttamalla iNOS-entsyymin hajoamista. Tämä saattaa olla merkittävä vaikutusmekanismi, jolla TGFβ suojaa IL-1:n aiheuttamalta rustotuholta. Osaltaan mekanismi osoittaa post-transkriptionaalisen säätelyn merkitystä typpioksidituoton säätelymekanismina transkriptionaalisen säätelyn ohella.

Ruston typpioksidisynteesin farmakologisen säätelyn tutkimuksissa mielenkiintoinen havainto oli, että artroosin hoidossa käytettävät anti-inflammatoriset steroidit eivät estäneet indusoituvaa typpioksidituottoa artroosirustossa ja rustosoluviljelmässä, vaikka ne estävät iNOS:n ekspressiota monissa muissa soluissa. Antireumaattisista lääkkeistä aurotiomalaatti ja hydroksiklorokiini estivät typpioksidituottoa artroosirustossa luultavasti transkriptiotekijä NF-κB:n aktivaation estosta johtuen. TNFα-antagonistit etanersepti ja infliximabi estivät TNFα:n aiheuttamaa typpioksidituottoa, mutta lääkeaineilla ei ollut vaikutusta IL-1:llä, IL-17:llä tai lipopolysakkaridilla käynnistettyyn typpioksidituottoon. Tuloksen mukaan TNFα ei välitä näiden välittäjäaineiden vaikutuksia tässä mallissa. Tulokset osoittavat, että aurotiomalaatti, hydroksiklorokiini, etanersepti ja infliksimabi estivät rustotuhoa edistävän typpioksidin tuottoa artroosirustossa ja siten saattaisivat toimia tulevaisuuden artroosin lääkehoitona. TNFα-antagonistit ja aurotiomalaatti estivät typpioksidituoton antireumaattisen lääkehoidon aikana *in vivo* saavutettavin pitoisuuksin, ja tämä on uusi tulehdusta estävä mekanismi näille lääkkeille nivelsairauksien hoidossa.

Introduction

Nitric oxide (NO) acts as a mediator in various physiological and pathophysiological processes in the human body. In the inflammatory reaction, NO generated by inducible nitric oxide synthase (iNOS) has both regulatory and proinflammatory effects. Interestingly, elevated levels of inflammatory mediators and enhanced NO production are found in osteoarthritic (OA) joints suggesting that OA is not a simple "wear and tear" phenomenon as thought earlier (Wollheim 2003).

The characteristic feature of OA is destruction of the articular cartilage, although pathognomonic features are found throughout the entire joint. The chondrocyte is the only cell type present in the cartilage and in the pathogenesis of OA chondrocytes play a central role by releasing inflammatory and destructive mediators. Proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) enhance the destructive processes in OA cartilage. Both of these cytokines are also capable of inducing the production of nitric oxide (NO) in chondrocytes. In fact chondrocyte was the second cell type in which human iNOS was cloned and characterized and that was carried out by Professor Moncada's research group in 1993 (Charles et al. 1993).

In inflamed joints, NO acts principally as a proinflammatory and destructive mediator. NO activates matrix metalloproteinases (MMPs), inhibits collagen and proteoglycan synthesis and induces chondrocyte apoptosis. NO is also involved indirectly in the upregulation of proinflammatory cytokine production in the inflamed joints by increasing the production of TNFα by the synovial cells (Goldring and Berenbaum 2004). In support of this view, a selective inhibitor of inducible NO synthase (iNOS), L-NIL, was found to slow down the progression of experimentally induced OA (Pelletier et al. 1998).

OA is the most common cause of musculoskeletal disability and pain in the Western world. In Finland, 5% Finnish men and 7% of women aged over 30 years suffer from clinically diagnosed knee osteoarthritis (Riihimäki and Heliövaara 2002). Presently approximately 14 000 hip or knee replacement operations are performed every year in Finland and the number of operations is on the increase (Nevalainen 2005). Current therapy of OA combines non-pharmacological and pharmacological therapy to relieve pain, maintain or improve joint function, and decrease disability (Jordan et al. 2003). At the moment there is no treatment

available that could prevent or retard the disease progression. As described above, NO production in chondrocytes is a promising target to inhibit destructive processes in the cartilage. The aim of the present study was to investigate regulation of iNOS expression and NO production in chondrocytes and in OA cartilage as potential target of drug development as well as to investigate if currently available disease-modifying antirheumatic drugs (DMARDs) could modify NO production in the cartilage.

Review of the literature

1 Osteoarthritis

Osteoarthritis (OA) is the most common cause of musculoskeletal disability and pain in the world. In the USA, only ischemic heart disease is a more common cause of work disability than OA in men over the age of 50. As a musculoskeletal disease, it accounts for more hospitalizations per year than rheumatoid arthritis (RA) (Dennison and Cooper 2003). According to the Health 2000 health examination survey in Finland (Riihimäki and Heliövaara 2002), 5% of Finnish men and 7% of women aged over 30 years suffer from clinically diagnosed knee OA. The prevalence of OA increases with age and 10% of Finnish men and 18% of women aged 65-74 years have symptomatic knee OA. Based on an interview survey, 20% of Finnish people report knee pain and over 10% have experienced enough discomfort to stop them walking due to knee symptoms during the past month (Riihimäki and Heliövaara 2002). In 2003, approximately 7000 hip and 7000 knee replacement operations were performed in Finland and the number of operations is steadily increasing (Nevalainen 2005).

Any synovial joint may be affected by OA, but it is most commonly found in the knee, hip, interphalangeal joints of the hand (distal interphalangeal, DIP and proximal interphalangeal, PIP), carpo-metacarpal (CMC) joint of the thumb, 1st metatarsophalangeal (MTP) joint, acromioclavicular joint and the cervical and lumbar spine. The MCP joints of the hand, wrist, glenohumeral joint, ankle, MTP and IP joints of the digits are less often and less severely affected (Figure 1). Certain risk factors like age, heredity, obesity, and gender can influence the overall predisposition to suffer OA, while mechanical factors like trauma, joint deformities, and repetitive stress can affect the localization and severity of OA (Dennison and Cooper 2003).

The principal symptom of OA is pain, which in the early stages of OA is related mainly to activity and weight bearing, but when the disease progresses pain is often present also at rest. Other clinical symptoms and signs include transient stiffness after inactivity, functional impairment, crepitus, swelling, instability, and deformity (Altman and Lozada 2003).

Current therapy of OA is symptomatic. There is no treatment available that could prevent or even retard the disease progression. The latest recommendations for management of osteoarthritis released by the European League Against Rheumatism (EULAR) (Jordan et al.

2003) and American College of Rheumatology (ACR) (Altman et al. 2000) underline individually tailored treatment combining non-pharmacological and pharmacological therapy to relieve pain, maintain or improve joint function, and decrease disability. Table 1 shows the EULAR recommendations for the management of knee OA (Jordan et al. 2003).

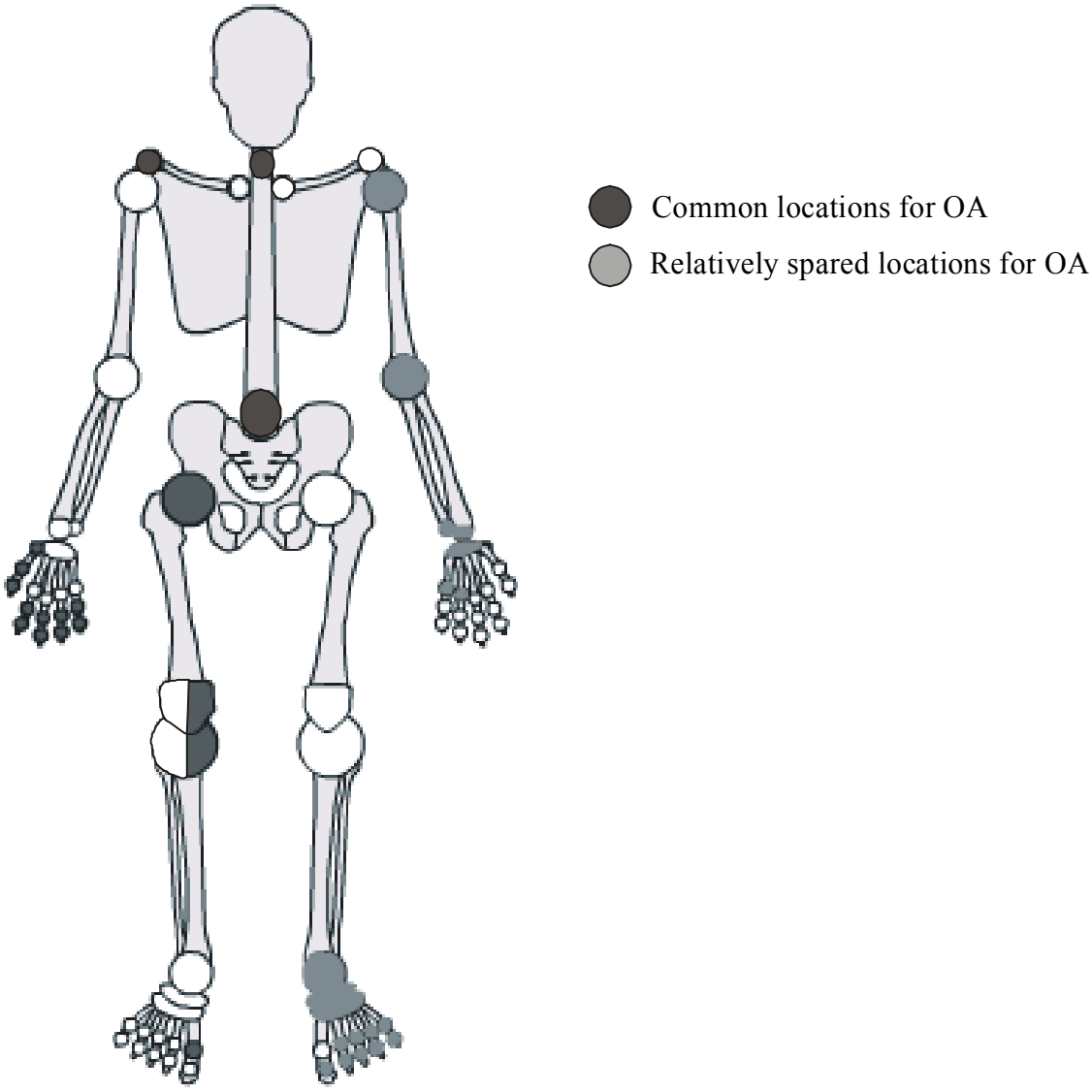


Figure 1. *The pattern of joint OA distribution. Knee, hip, interphalangeal joints of the hand (DIP and PIP), CMC joint of the thumb, 1st MTP joint, acromioclavicular joint and the cervical and lumbar spine are the most often affected joints. Other locations are less common.*

Table 1. *EULAR recommendations for the management of knee osteoarthritis based on both evidence and expert opinion.*

1. The optimal management of knee OA requires a combination of non-pharmacological and pharmacological treatment modalities.The treatment of knee OA should be tailored according to:
    a. Knee risk factors (obesity, adverse mechanical factors, physical activity)
    b. General risk factors (age, comorbidity, polypharmacy)
    c. Level of pain intensity and disability
    d. Signs of inflammation – for example, effusion
    e. Location and degree of structural damage
2. Non-pharmacological treatment of knee OA should include regular education, exercise, appliances (sticks, insoles, knee bracing), and weight reduction
3. Paracetamol is the oral analgesic to try first and, if successful, the preferred long term oral analgesic
4. Topical applications (NSAIDs, capsaicin) have clinical efficacy and are safe
5. NSAIDs should be considered in patients unresponsive to paracetamol. In patients with an increased gastrointestinal risk, non-selective NSAIDs and effective gastroprotective agents, or COX-2 selective NSAIDs should be used
6. Opioid analgesics, with or without paracetamol, are useful alternatives in patients whom NSAIDs, including COX-2 selective inhibitors, are contraindicated, ineffective, and/or poorly tolerated
7. SYSADOA (glucosamine sulphate, chondroitin sulphate, ASU, diacerein, hyaluronic acid) have symptomatic effects and may modify structure
8. Intra-articular injection of long acting corticosteroid is indicated for flare of knee pain, especially if accompanied by effusion
9. Joint replacement has to be considered in patients with radiographic evidence of knee OA who have refractory pain and disability

NSAID - nonsteroidal anti-inflammatory drug, COX-2 - cyclo-oxygenase 2, SYSADOA - symptomatic slow acting drugs for OA, ASU - avocado/soybean unsaponifiables. (Jordan et al. 2003)

1.1 Pathogenesis of osteoarthritis

The characteristic pathological feature of OA is destruction of the articular cartilage. The etiology of OA is unknown, but systemic risk factors like age, heredity, obesity, and gender influence on the suspectability to OA, while local biomechanical factors like trauma, joint deformities, and repetitive stress affect the site and severity of OA (Felson et al. 2000). Traditionally OA has been regarded as a consequence of mechanical wear and tear of aging cartilage. According to the present views, OA is instead considered as a local slowly progressing inflammatory disease, in which the chondrocyte plays an active role by releasing inflammatory and destructive mediators. In addition to the changes in cartilage, pathognomonic features are found in the entire joint including subchondral bone, ligaments, capsule, synovial membrane and periarticular muscles. There are no nerve endings in the cartilage and pain which is the main symptom in OA evolves from the changes in these noncartilaginous structures of the joint (Wollheim 2003, Altman and Lozada 2003).

1.1.1 Structural changes in cartilage

The normal adult articular cartilage matrix contains 60-80% of water, 10-20% of proteoglycans and 10-20% of collagen. Collagen is mostly (80-90%) of type II, the unique collagen type present in the cartilage. Water is attracted to the cartilage by negatively charged large proteoglycan aggregates, which adhere to the collagen scaffolding network. A balanced water content is required for normal cartilage function (Buchanan and Kean 2002).

Pathognomonic pattern of destruction in the OA-affected cartilage differs from the changes detected in aging cartilage (Table 2), but the changes detected in aging cartilage make it more vulnerable to the biomechanical factors like joint loading (Helminen et al. 2000). In the early stages of OA, water content of the cartilage increases probably due to weakening of the collagen framework. These changes together with a decrease in concentration, length and aggregation level of proteoglycans lessen the stiffness of cartilage structure. This results in fibrillation of the cartilage surface, ulcerations and formation of clefts. Clusters or clones of chondrocytes are formed around the clefts attempting to repair the changes by increased proteoglycan and collagen synthesis (Paukkonen and Helminen 1987). At this stage, the cartilage thickens, but the newly secreted matrix components are not normally organized. Finally, enhanced proteolytic degradation of the cartilage exceeds the enhanced matrix synthesis. Proteoglycan and collagen concentration decreases, cellularity of the cartilage reduces leading to exposure and eburnation of the subchondral bone (Buckwalter and Mankin 1997, Arokoski et al. 2001, Buchanan and Kean 2002, Wollheim 2003).

Table 2. *Structural changes in osteoarthritic vs. aging cartilage. Swelling of matrix caused by weakened collagen framework, chondrocyte hypertrophy and proliferation, and subchondral sclerosis are phenomena related to pathogenesis of osteoarthritis, and different from changes detected in aging cartilage. ⇑ = increase, ⇓ = decrease, ⇔ = no change.*

	Osteoarthritis	**Age related**
Cartilage hydration	⇑ (at early stages)	⇓
Proteoglycan concentration	⇓	⇔
Collagen concentration	⇓	⇔
Chondrocyte proliferation	⇑	⇔ or ⇓
Metabolic activity	⇑	⇔ or ⇓
Subchondral bone thickness	⇑	⇔

(Modified from Wollheim 2003)

Proteolytic degradation of cartilage is mediated by matrix metalloproteinases (MMPs). The MMP family contains more than 25 enzymes and they can be classified according to their major substrate targets (Table 3). The collagenases are able to cleave intact collagens and are suggested as rate-limiting in cartilage destruction (Mix et al. 2004). The activity of MMPs is endogenously regulated by tissue inhibitors of metalloproteinases (TIMPs). In OA, proinflammatory cytokines, IL-1 and TNFα, increase MMP expression and reduce TIMP expression shifting the balance towards cartilage destruction (Kobayashi et al. 2005). Some of the MMPs are able to degrade aggrecans, but aggrecanases are also involved in this degradative process. ADAM-TS4 and ADAM-TS5 (also called aggrecanase-1 and -2) are members of the ADAM-TS (a disintegrin and metalloproteinase with thrombospondin motifs) family, and they cleave aggrecan at specific cleavage site distinct from metalloproteinase cleavage site (Malfait et al. 2002, Wollheim 2003).

The loss of cartilage and following subchondral reactions lead to joint space narrowing, osteophyte formation, subchondral sclerosis and subchondral cysts (Figure 2). These late stage changes are also evident in radiographs taken from OA-affected joints. However, there is not a very good correlation between the radiographic changes and the symptoms of OA (Dennison and Cooper 2003).

Table 3. *Most common matrix metalloproteinases (MMPs) and their substrates related to arthritis. (The list is not comprehensive, e.g. membrane bound MMPs and elastases are not included in the table.)*

Group	MMP	Substrates
Collagenases		
Collagenase-1	MMP-1	Collagens I, II, II, IV, VII, VIII, X, XIV;
Collagenase-2	MMP-8	gelatin; aggrecan;
Collagenase-3	MMP-13	fibronectin; pro-MMP-9
Gelatinases		
Gelatinase-A	MMP-2	Gelatins; collagens III, IV, V, VII;
Gelatinase-B	MMP-9	fibronectin; laminin; proteoglycans;
		pro-MMP-13
Stromelysins		
Stromelysin-1	MMP-3	Aggrecan; fibronectin; gelatin;
Stromelysin-2	MMP-10	laminin; elastin; proteoglycans;
Stromelysin-3	MMP-11	collagen III, IV; pro-MMP-13

(Modified from Mix 2004)

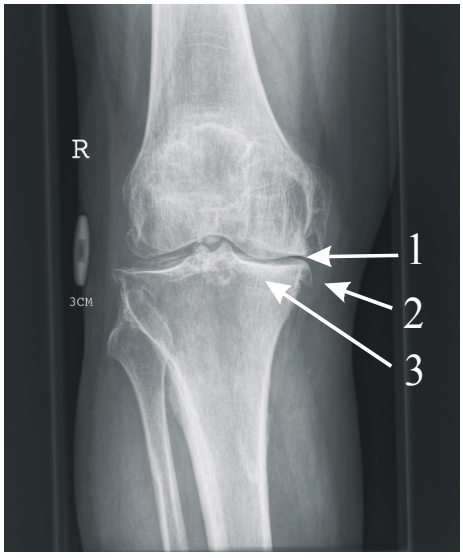


Figure 2. *Radiographic features of the OA-affected knee joint showing joint space narrowing (1), osteophyte formation (2) and subchondral sclerosis (3).*

1.1.2 Chondrocytes

Chondrocyte is the only cell type in the articular cartilage. Cells are sparsely dispersed in the abundant extracellular matrix and they form only 1% of the volume of the cartilage. Chondrocytes are located within lacunae, which are surrounded by metabolically active pericellular matrix or by a capsule and territorial matrix consisting of high concentrations of proteoglycans. The metabolically inert interterritorial matrix lies between these areas and composes 90% of the cartilage volume. There are no blood or lymph vessels, or nerves in the cartilage and synovial fluid supplies nutrients for the chondrocytes. The chondrocytes produce and maintain the surrounding matrix by synthesizing collagens, proteoglycans and regulators of cartilage metabolism. There are a few mitochondria, but the prominent endoplasmic reticulum and Golgi apparatus are characteristic to the chondrocytes and reflect the low oxygen consumption and high metabolic activity (Buchanan and Kean 2002, Wollheim 2003).

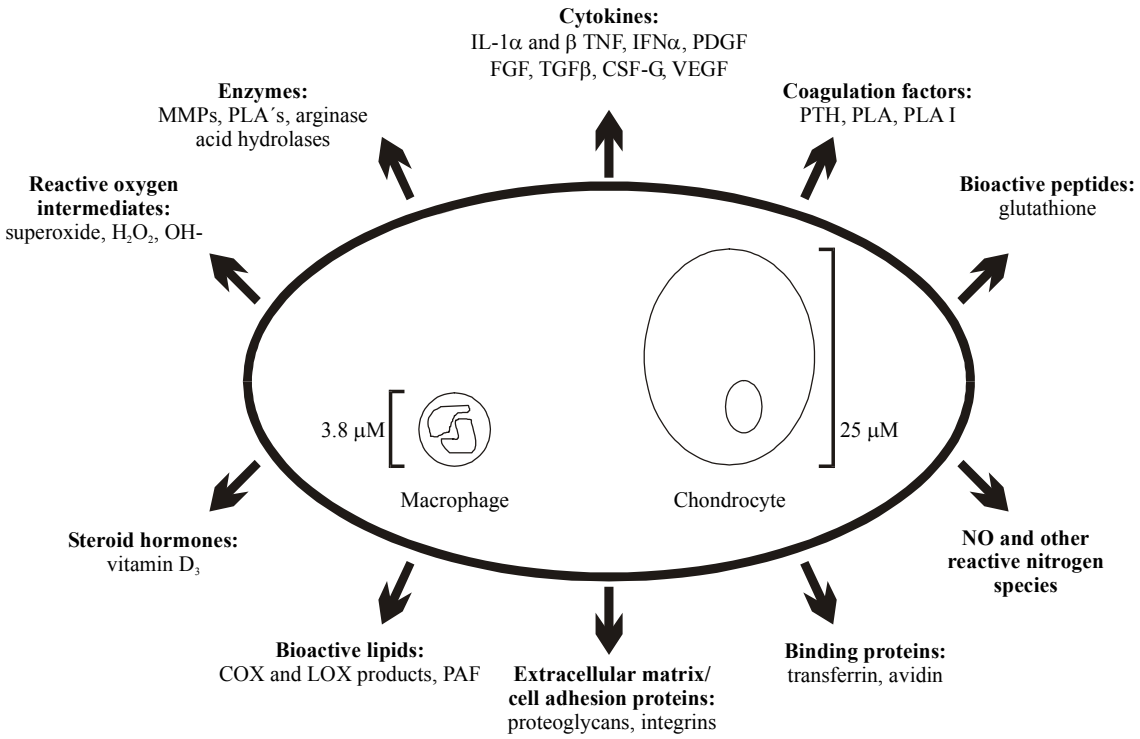


Figure 3. *Common inflammatory mediators released by OA-affected chondrocytes and activated macrophages. IL - interleukin, TNF - tumor necrosis factor, IFNα - interferon α, PDGF - platelet derived growth factor, FGF - fibroblast growth factor, TGFβ - transforming growth factor β, CSF-G - colony stimulating factor, VEGF - vascular endothelial growth factor , PTH - parathyroid hormone, PLA - phospholipase A, COX - cyclo-oxygenase, LOX - lipoxygenase, PAF - platelet activation factor, MMP - matrix metalloproteinase. (Modified from Attur et al. 2002)*

The chondrocytes respond to mechanical load (Kaarniranta et al. 1998), matrix alterations (e.g. fibronectin), and to factors (e.g. cytokines and growth factors) derived from the cartilage, synovial fluid or subchondral bone. In early OA, the chondrocytes attempt to repair the structural changes in cartilage and exhibit transiently enhanced matrix synthesis, proliferation and hypertrophy (Goldring 2000a). The gene expression profile in the OA-affected chondrocytes is changed and they release inflammatory mediators similar to activated macrophages (Figure 3) (Attur et al. 2002). Apoptotic cell death related to the local inflammation and matrix degradation further impairs the cartilage matrix integrity and mechanical strength (Yang C et al. 1997, Kühn et al. 2004).

1.1.3 Inflammation

Inflammation is a host defense response to injurious stimuli e.g. mechanical or chemical trauma and microbes and it is intended to destroy, dilute, isolate or repair the injurious agent or the injured tissue. At the site of inflammation, the blood vessels dilate resulting in increased local blood flow, permeability of the capillaries increases with consequent leak of fluids and plasma proteins in the interstitial spaces, and granulocytes and monocytes migrate into the tissues (Kumar et al. 2004).

The osteoarthritic joint lacks the cardinal signs of classical acute inflammation defined by Cornelius and Celsius - *rubor et tumor cum calore et dolore* - or redness and swelling with heat and pain. Although synovial swelling, tenderness and joint effusions, and even increases in serum C-reactive protein (CRP) may appear transiently during exacerbation phases of the disease, they reflect a secondary response to the disease rather than the primary pathogenetic event. Accordingly, OA has been traditionally classified as a degenerative joint disease caused by age-related wear and tear. Increasing knowledge about inflammatory components in the pathogenesis of OA expands this traditional concept of OA into a chronic but localized inflammation of the cartilage and surrounding tissues. However, the responsible initiator or promoter of these events is not known (Attur et al. 2002, Wollheim 2003, Kumar et al. 2004).

1.2 Cytokines in osteoarthritis

Inflammatory mediators, including cytokines and growth factors, are found in the synovial fluid in OA patients (Westacott et al. 1990, Schlaak et al. 1996). Cytokines and growth factors are produced by the chondrocytes, and synovial fibroblasts and macrophages and they act within the tissue or are released into the synovial fluid. In the cartilage, cytokines exert their effects on

chondrocytes in a paracrine, juxtacrine and autocrine fashion. In the normal joint, many of these mediators are present at low levels to maintain the homeostasis as the cartilage is continually remodeled by anabolic and catabolic processes. In OA, these processes are imbalanced resulting in gradual loss of articular cartilage. The course of the destructive process is determined by the balance between anabolic and catabolic mediators and their regulators, and by the local distribution of these mediators in the cartilage (Goldring and Goldring 2004).

Cytokines and growth factors involved in OA can be categorized according to their role in cartilage metabolism (Table 4). Proinflammatory cytokines, especially IL-1 and TNFα, act as central catabolic mediators. Cytokines that are able to regulate the activities of the other cytokines can be classified as modulatory. Cytokines that are able to block the actions of catabolic cytokines, such as the interleukin receptor antagonist (IL-1Ra), are regarded as being anti-catabolic. Growth factors that are able to enhance synthesis of extracellular matrix components, like transforming growth factor β (TGFβ), are considered as being anabolic (Goldring and Goldring 2004).

Table 4. *Best known cytokines involved in cartilage metabolism.*

Catabolic	**Modulatory**	**Anti-catabolic**	**Anabolic**
IL-1α and β	IL-6	IL-1Ra	TGFβ 1, 2, 3
TNFα	IL-11	IL-4	IGF-I
IL-17	LIF	IL-10	BMP 2, 4, 6, 7, 9, 13
IL-18		IL-13	
OSM			

IL - interleukin, TNFα - tumor necrosis factor-α, LIF - leukemia inhibitory factor, OSM - oncostatin-M, IL-1Ra - IL-1 receptor antagonist, IGF-I - insulin-like growth factor-I, TGFβ - transforming growth factor β and BMP - bone morphogenetic protein. (Goldring and Goldring 2004)

1.2.1 Interleukin-1

IL-1 is an important proinflammatory cytokine inducing fever, inflammation and tissue destruction, and in more severe cases, even in septic shock and death. With respect to the pathogenesis of inflammatory joint diseases IL-1 is thought to be the principal cytokine involved (Lotz 2001, Dinarello 2004). There are four primary proteins in the IL-1 superfamily, IL-1α, IL-1β (referred to as IL-1), IL-1 receptor antagonist (IL-1Ra) and IL-18 (Dinarello and Moldaver 2002). The regulation of IL-1 forms a complex network. Both IL-1α and IL-1β are produced as precursor molecules. The precursor form of IL-1α is able to bind and activate a cell

surface receptor. IL-1α is not commonly found in the circulation or body fluids, but it is able to function in an autocrine and even intracrine manner as suggested by a recent finding (Werman et al. 2004). In that study, overexpression of precursor IL-1α was shown to activate transcription factors nuclear factor κB (NF-κB) and activator protein-1 (AP-1) even in conditions when the IL-1 receptor is blocked. Unlike IL-1α, precursor IL-1β has to be cleaved to its active form by proteases such as the interleukin-1β converting enzyme (ICE or caspase-1) and then has to be transported out of the cell (Goldring 2000b, Dinarello and Moldaver 2002).

Two types of IL-1 receptors, type I (IL-1R I) and type II (IL-1R II), have been identified. They bind both IL-1α and β, but due to the intracellular nature of IL-α, IL-β is the primary ligand. The signal transduction of IL-1 is not transmitted directly through either of these receptors, but by a relatively stably expressed IL-1 receptor accessory protein (IL-1RAcP) that interacts only with type I receptor. Binding of IL-1 to IL-1R I recruits IL-1RAcP, and an active IL-1R signaling complex is formed that includes also the adaptor protein MyD88 and IL-1R-associated kinase (IRAK). IRAK activates TNF receptor-associated factor 6 (TRAF6), which interacts with the NF-κB inducing kinase (NIK). NIK phosphorylates and activates the inhibitory κB (IκB) kinase (IKK), which in turn phosphorylates IκB that is bound to NF-κB. NF-κB is released from IκB due to phosphorylation and that is followed by ubiquitination and degradation of IκB in the proteasome. NF-κB is now released and activated and it is able to translocate into the nucleus and to modify the transcription of target genes (Figure 4). In addition to the NF-κB pathway, also the tyrosine kinase pathway and the mitogen-activated protein kinase (MAPK) pathway may be involved in signalling (Lotz 2001, Dinarello and Moldaver 2002, Dinarello 2004).

Type II receptor acts as a "decoy" receptor with short cytosolic domain. Soluble forms of both type I (sIL-1R I) and type II (sIL-1R II) receptors have been reported. sIL-1R II binds to IL-1 with high affinity while sIL-1R I binds exclusively IL-1Ra. Thus the type II receptor, both membrane-bound and soluble, acts by downregulating the biological activity of IL-1 (Figure 4) (Fernandez-Botran 2000).

IL-1 receptor antagonist (IL-1Ra) is a specific receptor antagonist for IL-1α and IL-1β, because it is able to inhibit the biological activity of IL-1 by binding to IL-1 receptor type I with about the same affinity as IL-1α and β without activating signal transduction (Figure 4) (Hannum et al. 1990, Dinarello and Moldaver 2002). A 5-100-fold excess of IL-1Ra is needed to achieve 50% inhibition of the IL-1 activity (Arend et al. 1990, Smith et al. 1991).

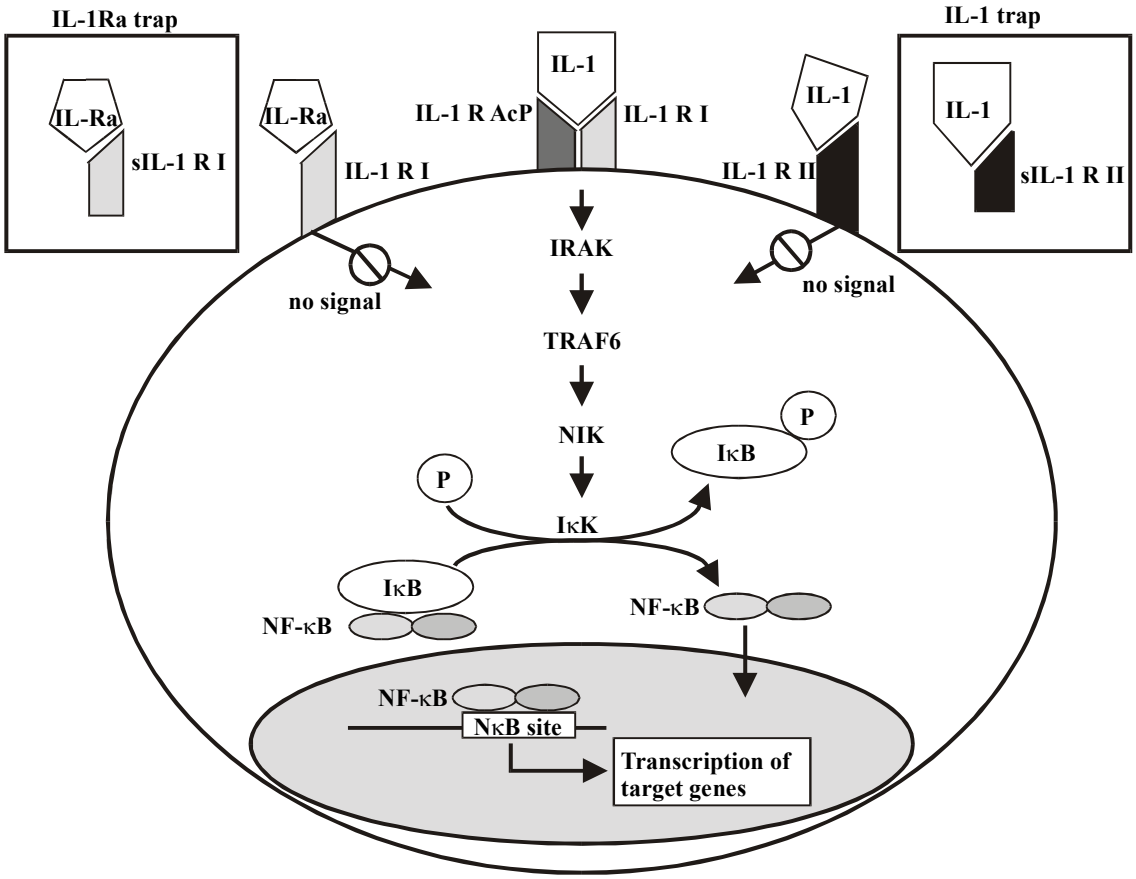


Figure 4. *Schematic representation of IL-1 signal transduction. The effect of IL-1 through IL-1 receptor type I (IL-1R I) and NF-κB pathway are presented in the middle. The function of membrane bound IL-1R II as a "decoy" receptor and of soluble IL-1R II as an IL-1 trap are presented on the right side. The function of IL-1Ra as a receptor antagonist and of soluble IL-1R I as an IL-1Ra trap are presented on the left side.*

In OA joints, IL-1 is produced by the chondrocytes (Melchiorri et al. 1998, Moos et al. 1999) as well as by the synovial membrane (Farahat et al. 1993, Smith et al. 1997), and elevated concentrations of IL-1 are found in the synovial fluid of OA patients (Wood et al. 1983, Westacott et al. 1990, Schlaak et al. 1996). In addition, the expression of ICE and IL-1R I is elevated in the OA-affected chondrocytes and synovial fibroblasts suggesting a high local activity of IL-1 and high sensitivity of the tissues to the effects of IL-1 (Martel-Pelletier et al. 1992, Saha et al. 1999).

In the pathogenesis of OA, IL-1 plays a central role in the destruction of articular cartilage. IL-1 inhibits the *de novo* synthesis of proteoglycan and type II collagen, and the proliferation of chondrocytes as well as inducing the production of matrix metalloproteinases and evoking chondrocyte death (Goldring 2000b, Lotz 2001). *In vivo* studies have confirmed that intra-articular injections of IL-1 induce synovitis and depletion of the cartilage matrix in animal

experiments (Pettipher et al. 1986, Chandrasekhar et al. 1990, Chandrasekhar et al. 1992, van Beuningen et al. 1993). The therapeutic use of IL-1Ra as a cytokine antagonist prevents the progression of experimental osteoarthritis (Pelletier et al. 1997). Anakinra, a recombinant human interleukin-1 receptor antagonist, was approved recently for the treatment of rheumatoid arthritis (RA) (Kwoh et al. 2002). In addition, ICE inhibitors and IL-1 Trap, a fusion protein containing two components of the cell surface IL-1 receptor and IgG1 constant fragment, have both gained favourable results in phase II RA trials (Dinarello 2004). As IL-1 is implicated in the pathogenesis of OA, inhibition of this cytokine is likely to have therapeutic effects. This was tested in a small, prospective, double-blind, clinical trial, where IL-1Ra was administered intra-articularly into the symptomatic knees of OA patients and was found to relieve pain for up to one month (Goupille et al. 2003). ICE inhibition could also have therapeutic potential in addition to the other IL-1 blockade approaches as inhibition of ICE reduced *in vitro* the production of the active form of IL-1β in OA cartilage and synovium (Saha et al. 1999). In fact, ICE inhibitor pralnacasan reduced *in vivo* joint destruction in two mouse models of OA (Rudolphi et al. 2003) and is now in clinical trials for OA (Wieland et al. 2005).

1.2.2 Tumor necrosis factor α

TNFα was first described as a macrophage derived product, which caused hemorrhagic necrosis of solid tumors. Since the biological activities of TNFα have been related also to the ability to induce cachexia, to initiate apoptosis, and to cause tissue injury and inflammation. The effects of TNFα in cartilage destruction are similar to or are synergistic with actions of IL-1 (Goldring and Goldring 2004). TNFα is synthesized as a precursor molecule that is cleaved to form active TNFα by the TNFα-converting enzyme (TACE). There are two receptors that mediate the effects of TNFα, type I receptor (TNFR I, also called the p55 receptor referring to its molecular weight of 55 kD) and type II receptor (TNFR II, also called the p75 receptor referring to its molecular weight of 75 kD). Both receptors trimerize upon activation by the TNFα ligand trimer. TNF receptor stimulation results in a complex process of signal transduction through caspases, protein kinase C (PKC), NF-κB and MAPK pathways and different cellular responses follow depending on the receptor type, expression of associated signaling machinery and interacting proteins (MacEwan 2002). Soluble forms of both type I (sTNFR I) and type II receptors (sTNFR II) are produced after the cleavage of the extracellular domains of these receptors. The soluble receptors are able to bind TNFα with the same affinity as the membrane bound forms of the receptors. The soluble receptors inactivate the effects of TNFα when the soluble receptor concentrations are high in relation to TNFα, but they carry TNFα to the cellular

receptors when low concentrations of both TNFα and the soluble receptors are present (Fernandez-Botran 2000, Dinarello and Moldaver 2002).

TNFα is present in low levels in the synovial fluid of OA patients (Hrycaj et al. 1995, Schlaak et al. 1996). TNFα is expressed in OA cartilage and to a lesser extent in OA synovial membrane (Melchiorri et al. 1998, Moos et al. 1999). Both receptor types can be found in the OA chondrocytes, but the level of TNFR I expression is elevated in OA chondrocytes and synovial fibroblasts when compared to nonarthritic cells (Westacott et al. 1994, Alaaeddine et al. 1997). In addition, OA-affected cartilage expresses upregulated TNFα and TACE mRNA levels when compared to the normal cartilage (Patel et al. 1998, Amin 1999).

In conjunction with IL-1, TNFα enhances the destructive processes in OA and is primarily involved in the onset of arthritis. *In vivo* injection of TNFα together with IL-1 results in more severe damage than injection of either cytokine alone suggesting a synergistic effect (Goldring 1999).

The TNFα-antagonists, infliximab, etanercept and adalimumab, have been recently introduced in the treatment of RA. These drugs bind to TNFα and block its bioactivity. Infliximab is a chimaeric monoclonal IgG1 antibody against human TNFα composed of constant human and variable murine regions. Etanercept is a fusion protein containing dimeric sTNFR II and a constant human IgG1 fragment, and adalimumab is a humanized IgG1 antibody against TNFα. Anti-TNFα therapy has evoked a significant reduction in disease activity and a retardation in joint erosions in controlled clinical trials in patients with RA (Camussi and Lupia 1998, Jarvis and Faulds 1999, Markham and Lamb 2000, Hochberg et al. 2003). Since TNFα is a cytokine which is crucially involved in the cartilage destruction in OA, TNFα blockade could offer therapeutic potential also in the treatment of OA.

1.2.3 Transforming growth factor β

The biological activities of TGFβ are primarily related to embryonal development and to wound healing, including control of cell proliferation, differentiation and extracellular matrix production. In normal cartilage, TGFβ regulates chondrocyte differentiation and matrix metabolism. In OA, TGFβ seems to play a role in matrix repair, although catabolic effects have also been reported (Goldring 2000a). Cytokines TGFβ 1, TGFβ 2 and TGFβ 3 are derived from different genes, but they share a set of common sequence and structural features. TGFβ is synthesized as a biologically inactive disulphide-linked dimer, which is cleaved to active cytokine dimer. Active TGFβ binds with a high affinity to the TGFβ type II receptor (TGFβRII) followed by recruitment of the TGFβ type I receptor (TGFβRI), and a ligand-receptor complex with ligand dimer and four receptors is formed. Related conformational changes reveal the

TGFβRII kinase activity leading to the phosphorylation and activation of TGFβRI. Consequently activated TGFβRI phosphorylates receptor-activated Smads (R-Smads) and releases them from the receptor complex to form a heterotrimeric complex of R-Smads and a common Smad4 and which is then translocated into the nucleus (Figure 5). In addition to the Smad-dependent pathway, TGFβ-signaling is mediated also through Smad-independent pathways like the MAPK pathways. The balance between these two pathways is believed to define cellular responses to TGFβ (Derynck et al. 1996, Derynck and Zhang 2003, Shi and Massague 2003).

Elevated levels of active TGFβ are found in the synovial fluid from OA patients (Fava et al. 1989, Schlaak et al. 1996). TGFβ 1, TGFβ 2, TGFβ 3 and receptors TGFβRI and TGFβRII are expressed in the human OA cartilage (Moos et al. 1999, Ayache et al. 2002).

TGFβ is an anabolic factor in cartilage and it enhances production of extracellular matrix proteins, stimulates chondrocyte proliferation and osteochondrogenic differentiation. In the OA process, TGFβ production is regarded mainly as a repair mechanism, but it may lead also to osteophyte formation and fibrosis. In addition, TGFβ has been shown to be a potent inducer of collagenase MMP-13 (Moldovan et al. 1997). In animal experiments, intra-articular injections of TGFβ as a short-term treatment (< 3 weeks) have been shown to result in enhanced extracellular matrix synthesis whereas as a long-term treatment (> 3 weeks) may augment cartilage destruction in healthy animals (Grimaud et al. 2002).

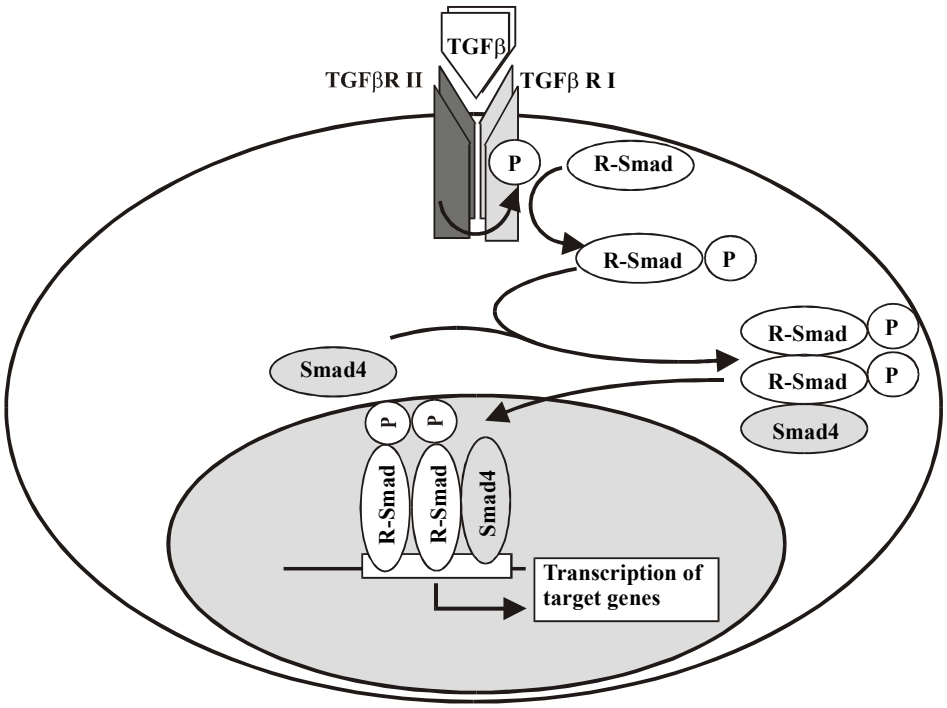


Figure 5. *Schematic representation of TGFβ signal transduction through Smad-pathway. R-Smad - receptor-activated Smad.*

2 Nitric oxide

Nitric oxide (NO) is a gaseous signaling molecule that mediates various physiological and pathophysiological responses in the human body, e.g. in the cardiovascular system, host defense and neurotransmission. The concept that NO could have significant biological effects was discovered by Murad and his research group in 1977, when NO and nitro compounds were shown to be able to activate guanylate cyclase (Arnold et al. 1977). Endogenous NO was found in 1987, when two research groups showed that the earlier described endothelium-derived relaxing factor (EDRF), a molecule released from the endothelium in response to acetylcholine (Furchgott and Zawadzki 1980), was NO (Palmer et al. 1987, Ignarro et al. 1987). After the initial findings, NO has been the focus of research, and knowledge about this novel molecule, its regulation and function has increased enormously. During the 1990s the importance of NO to biological sciences has been acknowledged: in 1992 the journal Science selected NO as "The molecule of the year" (Koshland, Jr. 1992) and in 1998 Robert F. Furchgott, Louis J. Ignarro and Ferid Murad received Nobel Prize in Physiology or Medicine "for their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system".

2.1 Biosynthesis of nitric oxide

NO is a short-lived, diffusible gas containing an unpaired electron in its structure. The NO molecule is synthesized from L-arginine and molecular oxygen (O_2) with the reaction being catalysed by a family of nitric oxide synthase (NOS) enzymes utilizes nicotineamide adenine dinucleotide phosphate (NADPH) as an electron donor. Enzymatically active NOS is a tetramer containing NOS as a dimer and two molecules of calmodulin (CaM), one at the C-terminal domain of each of the NOS proteins. The C-terminal reductase domain of NOS contains also tightly bound cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), while (6R)-tetrahydrobiopterin (BH_4) and iron protoporphyrin IX (haem) are bound to the N-terminal oxygenase domain of NOS protein (Alderton et al. 2001).

Intracellular L-arginine levels are important regulators of NO production once NOS is activated. L-arginine is synthesized from citrulline by the enzymes of the urea cycle, argininosuccinate synthase and argininosuccinate lyase and citrulline formed in NO production is thereby recycled back to L-arginine. The intracellular L-arginine concentration is regulated also by its transportation into cells via cationic amino acid transporters (CATs). CAT expression is induced by cytokines and LPS in some cell types. Arginase hydrolyses L-arginine to urea and L-ornithine and the activity is regulated usually in concordance with NO production, since the

intermediate product in NO synthesis, N-hydroxy-L-arginine (NOHA) inhibits arginase production thus maintaining substrate availability (Boucher et al. 1999, Mori and Gotoh 2000). In addition to the substrate availability, the limited availability of cofactors BH_4 or haem can impair NOS activity and NO production (Simmons et al. 1996).

In the enzymatic reaction NADPH is located in the reductase domain and it donates electrons (e^-) to FAD and further to FMN redox carriers, this reaction being controlled by CaM. Electrons then cross over to the other subunit of NOS and are transferred on to the haem at the oxygenase domain. The interaction with haem iron and BH_4 at the active site catalyzes the reaction of O_2 and L-arginine to form citrulline and NO in a two-phase reaction, where NOHA is formed as an intermediate (Figure 6) (Alderton et al. 2001).

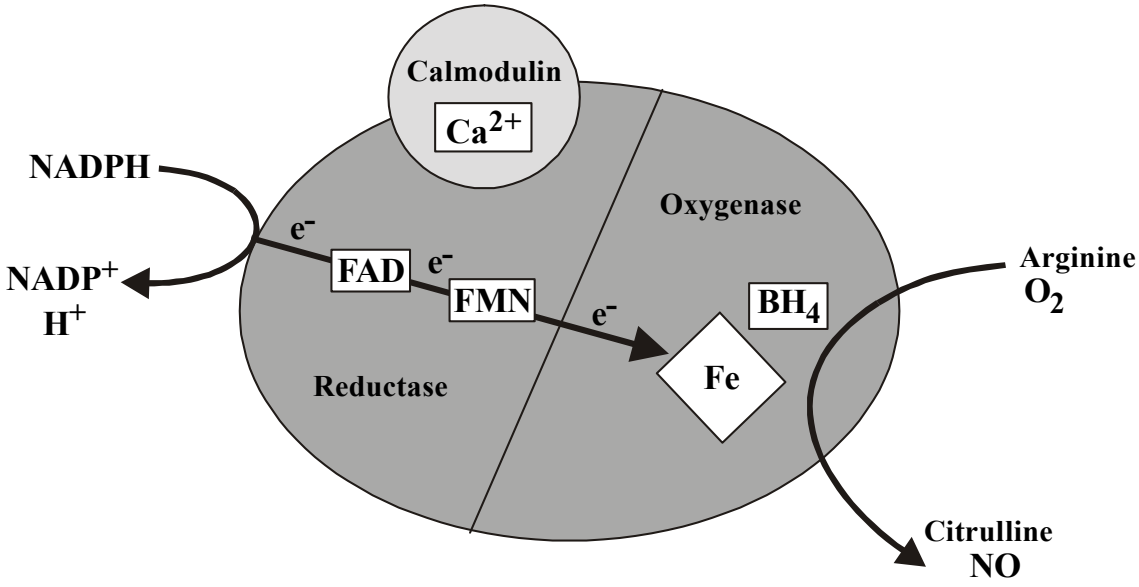


Figure 6. *NO production by NOS. NOS catalyzes the production of NO and L-citrulline from L-arginine, O_2, and NADPH-derived electrons. NOS requires FAD, FMN, calmodulin, haem and BH_4 as cofactors and prosthetic groups, when NO and citrulline are produced. (Alderton et al. 2001)*

The family of NOS enzymes consists of three subtypes encoded by different genes sharing ~50% homology between isoforms; neuronal NOS (nNOS, NOS I), endothelial NOS (eNOS, NOS III), and inducible NOS (iNOS, NOS II) (Table 5). Rat nNOS was the first purified and cloned isoenzyme and it was primarily found in neurons in the brain (Bredt et al. 1991). Subsequently human nNOS was found in neurons, and it is now known to be expressed also in other cell types e.g. in skeletal muscle and in airway epithelium (Nakane et al. 1993, Asano et al. 1994). Human eNOS was initially found in human vascular endothelium, and it is also expressed in other cell types e.g. in epithelial cells of airways, kidneys and reproductive organs,

in cardiac myocytes, platelets and hippocampus. Unlike the soluble enzymes, nNOS and iNOS, eNOS is membrane-associated in caveolae in plasma membrane by myristoylation and palmitoylation. Dissociation from caveolin activates eNOS (Janssens et al. 1992, Marsden et al. 1992, Shaul 2002). Both nNOS and eNOS are regarded as constitutive calcium-dependent enzymes, because they are mainly expressed constitutively in resting cells and produce low amounts of NO in response to an increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$), e.g. due to acetylcholine in endothelial cells, glutamate in brain, or collagen in platelets. The interaction of $[Ca^{2+}]_i$ and CaM is required for tight binding of CaM to constitutive NOS proteins and subsequent transient production of NO related to regulation of physiological processes in cardiovascular and nervous system (Alderton et al. 2001). Increased nNOS activity is associated with neurodegenerative diseases, convulsions and pain, while eNOS seems to have its main physiological role in the cardiovascular system (Salerno et al. 2002, Vallance and Leiper 2002).

iNOS was originally purified and cloned from a murine macrophage cell line, and the respective human enzyme was first isolated from primary human hepatocytes (Geller et al. 1993). Chondrocyte was the second cell type in which human iNOS was cloned and characterized by Professor Moncada's research group in 1993 (Charles et al. 1993). Subsequently, iNOS expression has been found in a variety of human cell-types and cell lines. Human iNOS cDNAs cloned from different cell types show > 99% sequence homology to human hepatocyte iNOS and about 80% sequence homology to murine macrophage iNOS. Species, strain and cell-type -dependent differences in the regulation of iNOS expression are believed to result mostly from differences in the regulation of iNOS transcription. Resting cells may express low-levels of iNOS, but activation by immunologic or inflammatory stimuli, like lipopolysaccharide (LPS), proinflammatory cytokines, hypoxia, foreign DNA or RNA, is usually needed to induce iNOS expression. In contrast to other species, most human cells require multiple synergistically acting cytokines to activate iNOS expression, but human chondrocytes, hepatocytes, and keratinocytes represent an important exception to that concept. Contrary to the constitutive isoforms of NOS, iNOS has tightly bound calmodulin even at low $[Ca^{2+}]_i$ and once iNOS is expressed, it results in extensive and prolonged NO production. NO production, via the iNOS pathway, plays both a protective role in host defense reaction as an antimicrobial agent that kills bacteria, viruses, protozoa, and tumor cells, and a detrimental role in inflammation as a reactive molecule that augments the reaction and mediates tissue damage (Vallance and Leiper 2002). Increased expression of iNOS has been implicated in the pathogenesis of a number of diseases or pathophysiological changes, including osteoarthritis, rheumatoid arthritis, asthma, inflammatory bowel disease, septic shock, malaria, malignancies, and multiple sclerosis (Moilanen et al. 1999, Rao 2000, Taylor and Geller 2000).

NOS gene family is dispersed in different chromosomes, nNOS is mapped to chromosome 12, eNOS to 7, and iNOS to 17. Expression of nNOS, eNOS, and iNOS splice variants resulting from alternative mRNA splicing has been detected. Regulation of splicing events and their biological significance is still unclear, e.g. the presence of tissue-specific variant NOS gene products or translational regulation by alternatively spliced mRNA products have been suggested (Park et al. 2000, Alderton et al. 2001, Newton et al. 2003). Recently, the relevance of polymorphic variations in NOS genes has been studied in relation to different diseases. In general, polymorphisms in the promoter region have the potential to modify transcription activity and thus enzyme expression levels, while variations in coding in the exon sequence may alter the primary structure of the protein and lead to functional changes. DNA variants of noncoding introns lead to normal functional enzyme, but may influence transcription levels or processing rates. Polymorphisms in the iNOS gene promoter, a single nucleotide substitution from G to C at position -969 and variations in the number of pentanucleotide CCTTT repeats, have been associated with severity of malaria (Burgner et al. 1998, Kun et al. 1998, Ohashi et al. 2002). Interestingly, polymorphisms in the number of CCTTT repeats in the iNOS promoter region seem to be linked also to the risk of rheumatoid arthritis, diabetic retinopathy, and intestinal type of gastric adenocarcinoma (Warpeha et al. 1999, Gonzalez-Gay et al. 2004, Tatemichi et al. 2005). eNOS polymorphisms have been identified in relation to cardiovascular diseases, diabetic nephropathy, and IgA nephropathy (Wang and Wang 2000, Kunnas 2002, Albrecht et al. 2003) and nNOS polymorphisms to asthma, infantile hypertrophic pyloric stenosis, and Parkinson's disease (Gao et al. 2000, Levecque et al. 2003, Saur et al. 2004).

Table 5. *Gene map locus and molecular weight (MW) of human nitric oxide synthase (NOS) isoforms.*

NOS	Gene map locus	MW (kDa)
iNOS	17cen-17q11.2	131
nNOS	12q24.2-12q24.3	161
eNOS	7q35-7q36	133

2.2 Effects of nitric oxide

Endogenous NO synthesized by different NOS isoforms regulates a variety of biological functions. In the cardiovascular system, NO maintains blood pressure, controls regional blood flow and limits platelet aggregation. Excess NO may result in hypotension e.g. in septic shock, whereas NO deficiency has been related to atherogenesis. The ability to produce high amounts of NO is needed for defense against bacteria, viruses, and parasites, but it may also cause tissue destruction in inflammatory diseases. NO acts as a neurotransmitter in the central nervous system and in peripheral NANC (non-cholinergic, non-adrenergic) nerves. Overproduction of NO has been related to neuronal destruction e.g. in cerebral ischemia, and lack of nNOS-derived NO may result in hypertrophic pyloric stenosis and erectile dysfunction (Änggård 1994, Moilanen et al. 1999, Davis et al. 2001).

The biological actions of NO are mediated by direct or indirect mechanisms depending on the microenvironmental conditions in which NO is produced and on the amount of NO produced, the latter relating to the NOS isoform responsible for NO production. At low concentrations of NO (< 1 μM), signaling is mediated mainly by direct mechanisms, involving an interaction of the NO molecule itself with metal complexes. At higher concentrations (> 1 μM), indirect effects dominate and signaling is mediated by reactive nitrogen species as a result of the interaction between NO and oxygen (O_2) or superoxide anion (O_2^-) (Davis et al. 2001).

Direct effects of NO are produced through formation of metal-nitrosyl complexes with biologically important transition metals iron (haem-containing proteins), copper or zinc. Soluble guanylate cyclase (sGC) is activated by this mechanism and it mediates many of the physiological effects of NO. Binding of NO to ferrous iron (Fe^{2+}) in the haem moiety of sGC results in a 400-fold increase in the synthesis rate of cGMP (3',5'-cyclic guanosine monophosphate), a second messenger of NO in a variety of physiological events e.g. relaxation of smooth muscle (Hanafy et al. 2001, Korhonen et al. 2005). Organic nitrates, like nitroglycerine, release NO after enzymatic hydrolysis, activate this pathway, and cause vasodilatation, which has been clinically used to relieve the symptoms of angina pectoris for over 130 years (Fung 2004). Other therapeutic targets in this pathway are phosphodiesterases (PDEs), especially PDE 5, which inactivate cGMP to 5'GMP. Selective inhibitors of PDE 5 enhance cGMP levels and augment NO-induced vasodilation e.g. in penile blood vessels, this being the mechanism of action of sildenafil and related drugs used in the treatment of erectile dysfunction (Rosen and McKenna 2002, Korhonen et al. 2005).

At high NO concentrations, the effects of NO are mediated indirectly through reactive nitrogen species. Depending on the redox state of oxygen, NO may undergo autoxidation to dinitrogen trioxide (N_2O_3) with molecular oxygen (O_2) or react with superoxide anion (O_2^-) to

form peroxynitrite ($ONOO^-$). If N_2O_3 is produced, it is readily hydrolyzed to nitrite (NO_2^-) and nitrosonium ion (NO^+), the latter being able to S-nitrosylate critical cysteine residues of proteins and by that mechanism it can modify their activity. S-nitrosylation has lately been appreciated as another important mechanism involved in the regulation of protein activity in addition to the well known protein phosphorylation pathways. The function of several transcription factors as well as kinases involved in signaling cascades, ion channels and metabolic proteins can be modulated via this mechanism. The specificity of S-nitrosylation signaling is achieved by subcellular vicinity of O_2, NO producing NOS and target proteins organized by scaffolding and anchoring proteins (Davis et al. 2001, Stamler et al. 2001, Hess et al. 2005, Korhonen et al. 2005).

Another high concentration effect of NO is mediated by the formation of peroxynitrite ($ONOO^-$) in the presence of equal amounts O_2^-. Superoxide is produced by mitochondria during aerobic respiration and by activated immune cells (macrophages and granulocytes) especially in conjugation with inflammation. Peroxynitrate is a highly reactive oxidant and nitrating agent, which is able to modify proteins, lipids and DNA. Excessive production of $ONOO^-$ is highly toxic since it causes nitration of proteins, inhibition of mitochondrial respiration, depletion of cellular energetics, DNA damage, apoptosis and cellular death (Denicola and Radi 2005). On the other hand, seemingly selective and reversible tyrosine nitration by $ONOO^-$ has been suggested to modify protein activity, cytoskeletal organization and to be involved in signal transduction. Identified substrates for post-translational protein modification by tyrosine nitration include prostacyclin synthase, cyclo-oxygenase, neurofilament L and manganese superoxide dismutase (Davis et al. 2001, Hanafy et al. 2001, Schopfer et al. 2003, Korhonen et al. 2005).

2.3 Inhibitors of nitric oxide synthases

Protein methyltransferases (PRMTs) form endogenous NOS inhibitors by methylation of arginine residues of proteins. Methylarginines N-monomethyl-L-arginine (L-NMMA) and asymmetric dimethylarginine (ADMA) are endogenously produced non-selective NOS inhibitors and are degraded by dimethylarginine dimethylaminohydrolases (DDAHs). NO is able to inhibit DDAH and thus to inhibit further NO production by preventing degradation of ADMA. Increased levels of ADMA have been related to cardiovascular diseases, but it is not yet known if ADMA is a marker rather than being a causal agent (Vallance and Leiper 2004).

Although NO is an important mediator of physiological responses, excessive NO production by nNOS and iNOS has been related to many pathological conditions. Selective

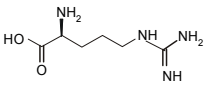
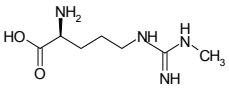
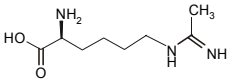
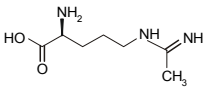
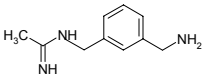
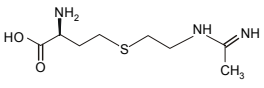
inhibitors of nNOS or iNOS in preference to eNOS represent potential drugs for various diseases and investigative tools to understand the effects of NO. Production of NO can be suppressed by inhibitors of NOS expression, but the classical NOS inhibitors act on enzyme activity most often by interfering with arginine binding sites. Alternative mechanisms include inhibition of dimerization, blockade of cofactors (BH_4, flavoprotein, CaM), and protein-protein interactions. Despite the fact, that the human isoforms share only about 50% homology in their primary sequence, the crystal structure of NOSs exhibits a high degree of structural similarity within the critical catalytic centre, which will pose a challenge to the development of isoenzyme selective NOS inhibitors. Some NOS inhibitor molecules tested *in vivo* for pharmacological use have also been rejected due to their poor cellular permeation, or incomplete bioavailability or due to toxic side effects (Alderton et al. 2001, Salerno et al. 2002, Vallance and Leiper 2002).

The first NOS inhibitors were modified analogues of the L-arginine substrate competing for the substrate binding site in the NOS enzyme. L-NMMA is a prototypic L-arginine analogue -type NOS inhibitor with no selectivity for the enzyme subtypes. Some of the L-arginine analogue -type NOS inhibitors have shown isoenzyme selectivity. N-nitro-L-arginine (L-NNA) and its methyl ester (N-nitro-L-arginine methyl ester, L-NAME) exhibit some selectivity towards constitutive enzymes, particularly for nNOS, whereas N-iminoethyl-L-lysine (L-NIL) and N-iminoethyl-L-ornithine (L-NIO) inhibit more potently iNOS than other NOSs. Improved iNOS selectivity has been achieved with amidine-derived inhibitors 1400W (Garvey et al. 1997), ONO-1714 (Naka et al. 2000), and GW274150 and GW273629 (Alderton et al. 2005). All these novel inhibitors compete with arginine and, with the exception of 1400W, do not exhibit acute toxicity making them suitable for animal studies. PPA250 is an example of an imidazole derivative, which inhibits the dimerization of iNOS (Ohtsuka et al. 2002) and 4-amino-H_4-biopterin, a BH_4 analogue -type NOS inhibitor, has shown iNOS selectivity in cell cultures (Werner et al. 2003). Table 6 shows iNOS selectivities of some experimentally used NOS inhibitors when compared to the constitutive isoforms (Alderton et al. 2001, Salerno et al. 2002).

In 1991 Petros et al. described case reports on initial administrations of NOS inhibitors to humans. Treatment with L-NMMA and L-NAME resulted in improved hemodynamics after unsuccessful conventional therapy in patients with septic shock (Petros et al. 1991). Although the initial reports and subsequent early clinical data were encouraging for NOS inhibitors in the treatment of septic shock, L-NMMA was reported to increase mortality in a prospective, randomized placebo-controlled trial after testing in almost 800 patients (Feihl et al. 2001). Interestingly, there was a higher proportion of cardiovascular deaths and a lower incidence of deaths caused by multiorgan failure in the L-NMMA group. The researchers speculated that this may be due to either overcorrection of vascular tone resulting in excessive increase in

ventricular afterload or a previously unrecognized primary effect of L-NMMA on myocardial performance (Feihl et al. 2001, López et al. 2004). Non-selective NOS inhibitor trials have been performed in patients with asthma (Taylor DA et al. 1998), migraine (Lassen et al. 1998), chronic tension-type headache (Ashina et al. 1999), psoriasis (Ormerod et al. 2000) and refractory cardiogenic shock (Cotter et al. 2003). Recently, inhibition of iNOS (and possibly nNOS) after oral administration of SC-51 (N-iminoethyl-L-lysine 5-tetrazole amide), prodrug of L-NIL, was reported to inhibit exhaled NO in healthy volunteers and patients with asthma throughout the 72 h assessment. A single dose was shown to be well tolerated with no effects on heart rate, blood pressure or airway obstruction (Hansel et al. 2003). Selective nNOS or iNOS inhibition may hold promise in the treatment of Alzheimer's disease, arthritis, asthma, cancer, cerebral stroke, glaucoma, inflammatory bowel disease, multiple sclerosis, OA, and Parkinson's disease as future therapeutics with benefits of targeted inhibition of NO production (Vallance and Leiper 2002, Neufeld 2004, Rao 2004, Bishop and Anderson 2005).

Table 6. *Isoform selectivity of NOS inhibitors. Selectivity is determined on the basis of IC50 values of inhibition of isolated NOS isoforms in similar experimental conditions. Caution should be taken when interpreting the values due to the following reasons: 1) selectivity studies performed with purified enzymes are not always comparable to in vivo conditions; 2) data given in table is extracted from two different studies.*

Inhibitor	Structure	Selectivity (fold) iNOS vs. nNOS	iNOS vs. eNOS
L-arginine			
L-NMMA		0.7	0.5
L-NIL		23	49
L-NIO		20	30-50
1400W		32	> 4000
GW274150		104	333

(Modified from Alderton et al. 2001)

3 Regulation of inducible nitric oxide synthase expression

In contrast to constitutive NOSs, iNOS is not generally present in resting cells but is induced in response to inflammatory and other stimuli. Constitutive isoforms, eNOS and nNOS, produce NO in response to elevated $[Ca^{2+}]_i$, which activates the enzyme by stabilizing the binding of calmodulin, whereas iNOS has tightly bound calmodulin independent of $[Ca^{2+}]_i$. Instead, NO production through the iNOS pathway is mainly regulated at the transcriptional and post-transcriptional levels affecting iNOS expression. Principally, when iNOS is expressed, it produces extensively NO until iNOS protein is degraded. Immunologic or inflammatory stimuli, like lipopolysaccharide (LPS), proinflammatory cytokines, hypoxia, foreign DNA or RNA induce iNOS expression in many inflammatory and tissue cells. In addition, fluid-induced shear stress has been shown to induce iNOS mRNA expression and NO production in human OA chondrocytes (Lee et al. 2002). Contrary to other species, most human cells require multiple cytokines acting synergistically to induce iNOS expression, but human chondrocytes, hepatocytes, and keratinocytes represent an important exception to that concept. Inter-species and cell-type differences probably result from the differences in the promoter region, signal transduction pathways or transcription factors expressed and activated. However, induction of iNOS transcription in both human and mouse cells seems to be dependent on activation of nuclear factor κB (NF-κB) and signal transducer and activator of transcription 1 (STAT1) pathways (Chu et al. 1998, Taylor and Geller 2000, Kleinert et al. 2003, Korhonen et al. 2005).

3.1 Transcriptional regulation

The human iNOS promoter region extends further upstream from the transcriptional start site than that of the murine iNOS. Based on deletion analyses, cytokine-inducible constructs of human iNOS 5'flanking region were found extending even up to -16.0 kb upstream from the transcription starting site when -1 kb was sufficient for murine iNOS promoter to show full functionality (Lowenstein et al. 1993, Xie et al. 1993, de Vera et al. 1996). The human iNOS promoter (Figure 7) contains a TATA box about 30 bp from the transcriptional start site, and regulatory elements NF-κB binding site (Kolyada et al. 1996), A-activator binding site (AABS) (Sakitani et al. 1998), Krüppel-like factor 6 binding site (KLF6) (Warke et al. 2003) and activator protein-1 (AP-1) binding site (Pance et al. 2002) are located within the first 1 kb. There is conflicting data concerning the importance of this area to the induction of iNOS transcription. Most studies have reported low basal promoter activity associated to that area, but no induction by cytokines (de Vera et al. 1996, Linn et al. 1997, Chu et al. 1998). Interestingly,

the same -1 kb human iNOS promoter fragment transfected to murine macrophages shows LPS and cytokine inducibility (Kolyada et al. 1996, Zhang et al. 1996). Transcription binding sites required for cytokine induction seem to be located upstream of position -3.8 kb (de Vera et al. 1996, Linn et al. 1997, Chu et al. 1998), probably even upstream of -7.0 kb (de Vera et al. 1996). This region contains regulatory elements for NF-κB (Marks-Konczalik et al. 1998, Taylor BS et al. 1998), AP-1 (Marks-Konczalik et al. 1998), gamma-activated site (GAS) (Ganster et al. 2001), and a negative regulatory element (NRE) (Feng et al. 2002).

The activation of NF-κB is critically needed in the induction of human iNOS gene. In resting cells, NF-κB proteins are present in the cytoplasm bound to inhibitors of NF-κB (IκBs). Activation by LPS, proinflammatory cytokines IL-1 and TNFα or other inducers activate the IκB kinase complex (IKK), which phosphorylates IκB. This results in ubiquitylation and subsequent proteasome degradation of IκB, and this subsequently releases NF-κB from its inhibitor. The NF-κB dimer is then translocated to the nucleus and bound to the NF-κB element to activate transcription of iNOS among other target genes (Li and Verma 2002). The human iNOS promoter contains multiple NF-κB binding sites. The NF-κB site at -8.2 kb is essential, whereas other sites also located upstream of -5 kb NF-κB enhance iNOS promoter activity in response to cytokines (Marks-Konczalik et al. 1998, Taylor BS et al. 1998). There is one proximal NF-κB binding site located at position -115 bp to -106 bp that may also control the human iNOS promoter activity (Marks-Konczalik et al. 1998, Sakitani et al. 1998, Kolyada and Madias 2001), although no role for induction of transcription has also been reported (Taylor BS et al. 1998).

Interferon γ (IFNγ) is an important inducer of iNOS expression in most cell types. Activated Stats together with p48 may form an interferon stimulated gene factor 3 (ISGF3), which binds to the IFN-stimulated response element (ISRE) to activate the IFN-inducible genes. IFNγ may also activate receptor associated Janus-activated kinase (Jak) resulting in phosphorylation of STAT1 tyrosine residue. Activated STAT1s are released from the receptor, dimerized and translocated to nucleus (Kisseleva et al. 2002). STAT1s bind to GAS, and two sites associated with regulation of human iNOS transcription have been identified at -5.8 kb and -5.2 kb. The upstream site is a bifunctional composite NF-κB/STAT1 binding site and may require binding of both NF-κB and STAT1 in a protein-protein-DNA complex for promoter activity (Ganster et al. 2001). One of the critical target genes of GAS is IFN regulatory factor (IRF-1), which has a cell type-dependent role in iNOS transcription. Macrophages derived from IRF-1 gene knockout (KO) mice are not able to express iNOS in response to LPS (Martin et al. 1994), while chondrocyte responses to IFNγ and LPS or IL-1β remain intact (Shiraishi et al. 1997).

In addition to NF-κB and STAT1, other reported positive regulators of human iNOS promoter activity include CAAT/enhancer binding protein β (C/EBPβ, binding to AABS) (Sakitani et al. 1998) and KLF6 (Warke et al. 2003). The transcription factor AP-1 seems to have both positive and negative effects in the regulation of human iNOS expression (Marks-Konczalik et al. 1998, Pance et al. 2002). In bovine articular chondrocytes, AP-1 does not participate in the transcriptional regulation of the iNOS gene induced with IL-1 (Mendes et al. 2003), whereas as in the human iNOS promoter, the proximal AP-1-like site regulates negatively basal and inducible iNOS transcription while the -5.3 kb site seems to be essential for iNOS promoter activity (Marks-Konczalik et al. 1998, Pance et al. 2002). Binding of NF-κB repressing factor (NRF) to NRE constitutively suppresses the basal transcription of the human iNOS gene (Feng et al. 2002).

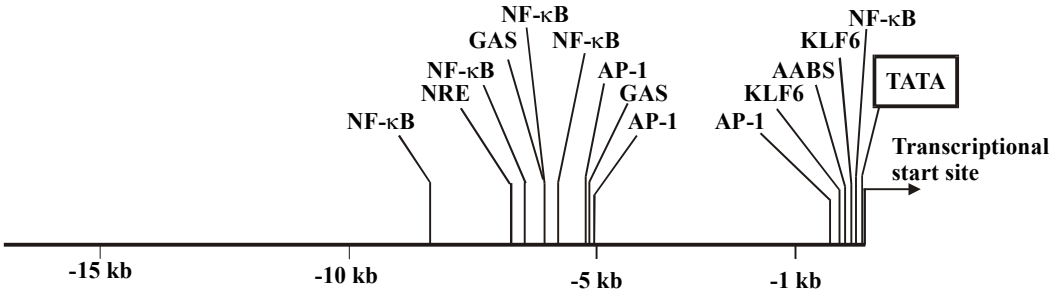


Figure 7. *Schematic representation of human iNOS promoter. Relative positions of regulatory elements NF-κB, KLF6 - Krüppel-like factor 6, AABS - A-activator -binding site, AP-1 - activator protein-1, GAS - gamma-activated site and NRE - negative regulatory element are shown. (Modified from Kleinert 2003)*

3.2 Post-transcriptional regulation

iNOS expression and NO production are regulated also at the post-transcriptional level. Evidence of post-transcriptional iNOS regulation has been found related to mRNA stability, efficiency of mRNA translation and protein degradation. These post-transcriptional regulatory mechanisms and their importance in iNOS regulation are not yet fully understood (Kleinert et al. 2003, Korhonen et al. 2005).

Unstimulated cells may show significant basal activity of iNOS promoter constructs and continuous iNOS transcription, while iNOS mRNA and protein expression or NO synthesis is not detected. Therefore it is likely that unstimulated cells produce highly unstable iNOS mRNA (de Vera et al. 1996, Laubach et al. 1997, Linn et al. 1997, Rodríguez-Pascual et al. 2000). Based on sequence analysis, both human and murine iNOS 3'-untranslated region (3'-UTR) contain adenosine-uridine (AU) -rich elements (AREs) (Lyons et al. 1992, Geller et al. 1993), that are known to destabilize the mRNAs of transiently expressed proteins. AU-binding proteins

may either promote or prevent exonucleolytic degradation of mRNA by the exosome protein complex and thus refine the effect of AREs on mRNA stability (Zhang et al. 2002). The involvement of iNOS 3'-UTR in the induction of iNOS expression was reported in an experiment, in which a gene construct containing both the promoter and the 3'-UTR of the human iNOS exhibited higher reporter gene activity than the control without 3'-UTR (Nunokawa et al. 1997). Studies with the protein translation inhibitor, cycloheximide, show increased murine iNOS mRNA stability suggesting that mRNA stability is regulated by factors synthesized *de novo* or in connection to translation (Evans et al. 1994, Hattori and Gross 1995, Park and Murphy 1996, Korhonen et al. 2002). AU-binding proteins like tristetraprolin (TTP), KSRP and AUF1 bind to AREs of mRNA of transiently expressed genes and recruit the exosome to degrade the mRNA (Chen et al. 2001, Mukherjee et al. 2002), while HuR stabilizes mRNA (Brennan and Steitz 2001). HuR, a member of Hu family of RNA-binding proteins, has been shown to bind to 3'-UTR of iNOS mRNA and to stabilize it (Rodríguez-Pascual et al. 2000). $[Ca^{2+}]_i$ elevating agents have been reported to inhibit NO production via reduced mRNA stability in IL-1 -stimulated human articular chondrocytes and in LPS-activated J774 macrophages (Geng and Lotz 1995, Korhonen et al. 2001). Other factors that have been shown to regulate iNOS by enhanced iNOS mRNA degradation include TGFβ (Vodovotz et al. 1993), 8-bromo-cGMP (Pérez-Sala et al. 2001), dexamethasone (Korhonen et al. 2002), and Jnk-inhibitor SP600125 (Lahti et al. 2003), while treatment with forskolin (adenylate cyclase activator) (Kunz et al. 1994), dibutyryl cAMP (Oddis et al. 1995), BH_4 (Linscheid et al. 1998), isoproterenol (β-sympathomimetic drug) (Gustafsson and Brunton 2000), and SB220025 (p38 inhibitor) (Lahti 2004) stabilize iNOS mRNA.

NO production can also be modulated at the translational level. In human cardiac myocytes, cytokines were shown to induce iNOS mRNA expression, while iNOS protein or NO synthesis was not detected (Luss et al. 1997). Similarly, dexamethasone (Walker et al. 1997), sodium salicylate (Sakitani et al. 1997) and cytokines TGFβ (Vodovotz et al. 1993) and IL-13 (Bogdan et al. 1997) have been shown to act at the translational level. Recently, IL-13 was shown to increase arginase activity, which resulted in L-arginine depletion and reduced iNOS mRNA translation (El-Gayar et al. 2003). Confusingly, it is known that maximal iNOS activity and NO production cannot be achieved with intracellular L-arginine concentrations sufficient to saturate the enzyme, a phenomenon called the "arginine paradox" (Kurz and Harrison 1997). This may be explained by the finding that relative intracellular L-arginine depletion reduces iNOS mRNA translation by inactivating eukaryotic initiation factor2α, an important factor in initiation of translation in eukaryotes (Lee et al. 2003). It is not known if this mechanism applies to dexamethasone, salicylates or TGFβ, but it is noteworthy that at least dexamethasone (Gotoh et al. 1997) and TGFβ (Boutard et al. 1995) have been shown to activate arginase.

After iNOS protein is expressed, it may be directed to enhanced degradation under certain conditions. The concept of regulation of iNOS expression at the level of iNOS protein stability was trigged by reports showing that TGFβ (Vodovotz et al. 1993) and dexamethasone (Kunz et al. 1996) may induce their inhibitory effect on NO production through enhanced iNOS protein degradation though other mechanisms may be involved. The calpain and proteasome pathways are the major proteolytic pathways in the degradation of iNOS protein (Kone et al. 2003). Dexamethasone was shown to promote proteolytic degradation of iNOS in IFNγ-stimulated RAW macrophages and using cystein protease calpain I inhibitor (N-acetyl-Leu-Leu-nor-leucinal) the enhanced proteolysis could be blocked (Walker et al. 1997, Walker et al. 2001). Overexpression of caveolin-1 was shown to decrease iNOS protein levels in cytokine-treated cells, while iNOS mRNA levels remained unaltered. Addition of the proteasome inhibitor, lactacystin, enhanced iNOS protein levels pointing to a contribution of the proteasome pathway in iNOS protein degradation of caveolin-1 transfected cells (Felley-Bosco et al. 2000). Enhancement of iNOS protein expression was also detected in human kidney HEK293 cells with stable iNOS expression and in cytokine-induced epithelial RT4 and murine RAW 264.7 macrophages after treatment with the proteasome inhibitor lactacystin (Musial and Eissa 2001). iNOS seems to be targeted for proteasome degradation by ubiquitylation of the protein (Kolodziejski et al. 2002).

In addition to the substrate availability, a limited availability of cofactors BH_4 or haem can impair iNOS activity and NO production (Albakri and Stuehr 1996, Simmons et al. 1996) and they all also promote and/or stabilize the active dimeric form of NOSs (Alderton et al. 2001, Blasko et al. 2002). Protein modifications may also regulate iNOS activity. For instance, tyrosine nitration by peroxynitrite was reported to decrease iNOS activity, pointing to a negative feedback loop preventing overproduction of NO (Lanone et al. 2002).

4 Nitric oxide in osteoarthritis

Markers of enhanced NO production are found in synovial fluids, serum and urine from patients with OA and RA, suggesting a role for NO in these joint diseases. Elevated concentrations of nitrite (a metabolite of NO) (Farrell et al. 1992, Renoux et al. 1996, Holm 2000, Karan et al. 2003), nitrotyrosine (a marker of NO dependent oxidative damage) (Kaur and Halliwell 1994), S-nitrosoproteins (nitrosylated derivatives of molecules containing sulfhydryl groups) (Hilliquin et al. 1997) and N-hydroxy-L-arginine (an intermediate of NO synthesis) (Wigand et al. 1997) have been found in serum or synovial fluid from OA and RA patients. Treatment with the anti-

inflammatory corticosteroid prednisolone has been reported to decrease the elevated serum concentrations of S-nitrosoproteins (Hilliquin et al. 1997) and urinary concentrations of nitrate in RA patients (Stichtenoth et al. 1995). Anti-TNFα therapy has also been shown to reduce iNOS expression and NOS enzyme activity in peripheral blood mononuclear cells and this correlates with changes in the number of tender joints in treated RA patients (Perkins et al. 1998).

Expression of iNOS in OA cartilage and synovium has been shown by immunohistochemistry (Sakurai et al. 1995, McInnes et al. 1996, Grabowski et al. 1997). Enhanced expression of iNOS principally in chondrocytes and significantly less in synovial cells in OA patients suggests that cartilage is the major source of NO in OA joints (Melchiorri et al. 1998). In RA joints, iNOS expression has been demonstrated predominantly in synovial lining cells, endothelial cells and chondrocytes, and to a lesser extent in infiltrating mononuclear cells and synovial fibroblasts (Sakurai et al. 1995). Macrophages in the pseudo-synovial membrane around the loosened joint implants have also been reported to express iNOS (Moilanen et al. 1997). Positive nitrotyrosine staining has been detected in synovial samples of OA and RA patients (Sandhu et al. 2003).

Induction of NO synthesis in rabbit articular chondrocytes was first demonstrated in 1991 (Stadler et al. 1991) and two years later this was extended to human chondrocytes (Palmer et al. 1993, Charles et al. 1993, Maier et al. 1994). In addition to the 131 kDa human iNOS that was first found and cloned in human chondrocytes, another NO synthase was described by Amin et al (1995) in human OA cartilage. This enzyme, called OA-NOS, was reported to be different from iNOS and relatively close to nNOS in its molecular weight and antibody binding. The sequence of OA-NOS was not reported and it has not been determined if it is a novel isoform of NOS or a modification of one of the earlier documented isoforms, either iNOS or nNOS (Amin et al. 1995).

NO seems to be a proinflammatory and destructive mediator in cartilage (Figure 8). NO has been reported to activate matrix metalloproteinases (MMPs) (Murrell et al. 1995) and cyclo-oxygenase (de Mello et al. 1997, Nédélec et al. 2001), inhibit collagen (Cao et al. 1997) and proteoglycan synthesis (Häuselmann et al. 1994, Taskiran et al. 1994, Järvinen et al. 1995), increase susceptibility to injury by other oxidants (e.g. H_2O_2) (Clancy et al. 1997) and induce chondrocyte apoptosis (Blanco et al. 1995, Hashimoto et al. 1998). NO has been shown to shift the cytokine balance towards the proinflammatory and destructive direction by reducing the synthesis of anabolic mediator transforming growth factor β (TGFβ) (Studer et al. 1999), by contributing to resistance against anabolic effects of IGF-I (Studer et al. 2000, Loeser et al. 2002) and by increasing the production of catabolic mediator TNFα by synovial cells (McInnes et al. 1996).

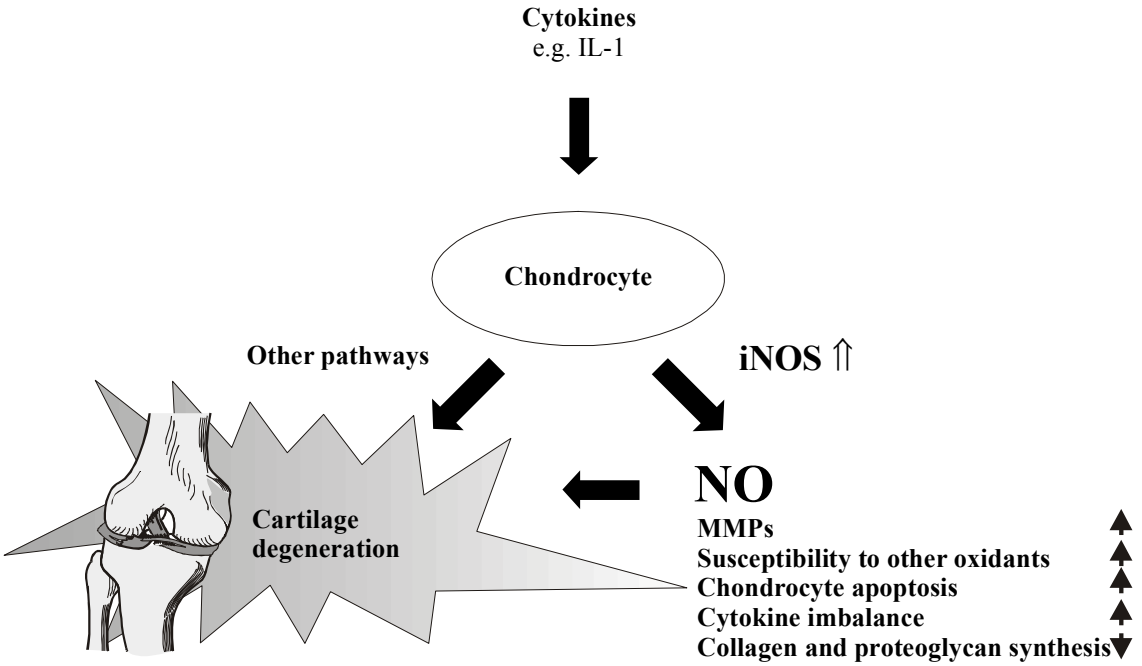


Figure 8. *The effects of NO in OA cartilage.*

4.1 Nitric oxide synthase inhibitors in experimental models of inflammatory arthritis and osteoarthritis

Since they may be potential novel drugs for treatment of inflammatory joint diseases, NOS inhibitors have been studied in experimental models of arthritis. Administration of non-selective NOS inhibitors L-NAME and L-NMMA have been reported to reduce the clinical and histological signs in arthritis induced by injection of streptococcal cell wall fragments (SCW) (McCartney-Francis et al. 1993), in adjuvant arthritis (Ialenti et al. 1993, Oyanagui 1994, Stefanović-Račić et al. 1994), in spontaneous autoimmune arthritis in MRL lpr/lpr mice (Weinberg et al. 1994), and in antigen-induced arthritis (de Mello et al. 1997). Similar results have been obtained from studies using selective iNOS inhibitor L-NIL in adjuvant induced arthritis (Connor et al. 1995), highly selective iNOS inhibitor GW274150 in collagen-induced arthritis (Cuzzocrea et al. 2002), and the inhibitor of the dimerization of iNOS PPA250 in collagen-induced arthritis in the mice and in adjuvant arthritis in the rat (Ohtsuka et al. 2002). In addition, there are also studies showing that iNOS inhibition with L-NIL has exacerbated the acute inflammatory response in antigen-induced arthritis (Veihelmann et al. 2002) and the chronic inflammatory response in SCW-induced arthritis (McCartney-Francis et al. 2001).

The role of NO in arthritis has been evaluated also in experimental arthritis models in iNOS knockout (KO) mice. Similarly to the situation with NOS inhibitors, the literature reports controversial findings. In iNOS KO mice, arthritis manifestations have been reported to be milder in collagen-induced and autoantibody mediated arthritis (AMA) (Cuzzocrea et al. 2002, Kato et al. 2003), whereas no differences were detected in MRL-lpr/lpr or Lyme arthritis models (Gilkeson et al. 1997, Brown and Reiner 1999), and exacerbation was reported in antigen-induced and septic arthritis following *Staphylococcus aureus* infection (McInnes et al. 1998, Veihelmann et al. 2001). Interestingly, van de Loo et al. reported cartilage protection in zymosan-induced gonarthritis of iNOS KO mice while the joint inflammation remained unaffected (van De Loo et al. 1998). These controversial findings obtained from studies with NOS inhibitors and iNOS KO mice may reflect differences in iNOS expression in these heterogeneous models of arthritis depending on the etiology, relating to a certain phase in the pathogenesis of the experimental models or associating to the location in joint. Lack of iNOS gene may result in unpredictable compensatory effects during embryonic development or in adult mice and thus affect susceptibility to arthritis as compared to pharmacological iNOS inhibition in wild type mice.

There are only few studies on the effects of NO in experimental models of OA. Pelletier et al. reported that a selective inhibitor of iNOS, L-NIL, was able to slow down the progression of instability induced OA in dogs (Pelletier et al. 1998). L-NIL treatment reduced the macroscopically evaluated incidence and size of osteophytes and decreased cartilage lesions, and attenuated the histological severity of cartilage lesions and synovial inflammation. In further studies with this model L-NIL has evoked a reduction in MMP activity, a reduced level of chondrocyte apoptosis possibly mediated by a decrease in the level of caspase-3 expression and decreased ICE expression in cartilage (Pelletier et al. 1998, Pelletier et al. 1999, Pelletier et al. 2000, Boileau et al. 2002).

Development of OA in iNOS KO mice has been studied in two experimental OA models. iNOS deficiency has been reported to prevent the degree of cartilage destruction and osteophyte formation in OA model induced with intra-articular collagenase injection into the knee joint (van den Berg et al. 1999). An opposite result was reported when OA was induced with transection of the medial collateral ligament and partial medial meniscectomy resulting in accelerated development of OA lesions in the cartilage of iNOS KO mice (Clements et al. 2003).

Taken together, NO seems to be a destructive mediator in cartilage, although as with the other inflammatory mediators, it may also evoke protective effects. The role of NO in OA may be connected to a certain phase in the development of cartilage destruction, to the etiological factors of OA or to the site of pathologic process. The whole biological network in the

pathogenesis of OA involves multiple mediators and inhibition of NO may produce a number of changes some of which may be unpredictable. However, the present knowledge we have points to therapeutic value for iNOS inhibitors in the treatment of OA.

Aims of the study

The aim of the present study was to investigate regulation of iNOS expression and NO production in chondrocytes and in OA cartilage as potential targets of drug development as well as to investigate if currently available disease-modifying antirheumatic drugs (DMARDs) can affect NO production, which could be involved in their therapeutic effect.

The detailed aims were:

1. to study regulation of iNOS expression and NO production in human OA cartilage and in murine H4 chondrocytes (I, IV)
2. to investigate the regulation of NO production in OA cartilage by endogenously produced cytokine antagonists (II, III)
3. to study the effects and mechanisms of action of TGFβ on NO production in chondrocytes (IV)
4. to investigate if currently available disease-modifying antirheumatic drugs (DMARDs) affect on NO production in chondrocytes and in OA cartilage (V)

Materials and methods

1 Tissue and cell cultures

Cartilage tissue was obtained from the leftover pieces of total knee replacement surgery from patients with OA (I, II, III, V) or RA (III). The study was approved by the ethics committee of Tampere University Hospital. Cartilage samples were washed with phosphate buffered saline (PBS) and processed for the experiments within a few hours after the operation. Full thickness pieces of articular cartilage from femoral condyles, tibial plateaus and patellar surfaces showing macroscopical features of early OA were removed aseptically from subchondral bone with a scalpel and cut into small pieces. These pieces of cartilage were incubated at 37°C in humidified 5% carbon dioxide atmosphere in Dulbecco´s modified Eagle´s medium (DMEM) with glutamax-I containing 10% heat-inactivated fetal bovine serum purchased from Gibco BRL, Paisley, UK (I-III) or from Cambrex Bioproducts Europe, Verviers, Belgium (IV-V), penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml) obtained from Gibco BRL, Paisley, UK (I-III) or from Invitrogen, Carlsbad, CA, USA (IV-V). J774 murine macrophages (American Type Culture Collection, Rockville, USA) were used as the reference cell line in some studies. J774 cells were cultured under similar conditions and harvested with trypsin-EDTA (ethylenediaminetetraacetic acid; Gibco BRL, Paisley, UK).

The murine chondrocyte cell line H4 (van Beuningen et al. 2002) was kindly provided by Dr van Beuningen and Prof van der Kraan, University Medical Center, Nijmegen, The Netherlands. Chondrocytes were cultured at 37°C in humidified 5% carbon dioxide atmosphere in DMEM and Ham's F-12 medium both with L-glutamine (Cambrex Bioproducts Europe, Verviers, Belgium) (1:1, v/v) and supplements as given above.

Cartilage pieces were placed on 24-well plates for nitrite measurements and for Western blot assays. Cells were seeded on 24-well plates for nitrite measurements, on six-well plates for Western blot and real-time polymerase chain reaction (PCR) assays, and on 10 cm dishes for EMSA experiments. Cells were grown to confluence for 72 h (J774 macrophages) or 24 h (chondrocytes) prior to experiments.

2 Nitrite assays

Concentrations of nitrite, a stable product of NO in aqueous solutions, were measured in the culture medium with the Griess reaction. Briefly, culture medium (100 µl) was incubated with 100 µl of Griess reagent (0.1% naphthalethylenediamine dihydrochloride, 1% sulphanilamine, 2.5% H_3PO_4). Nitrite reacts with Griess reagent and forms purple azo dye. The absorbance was measured spectrophotometrically at 540 nm. The concentration of nitrite was calculated using sodium nitrite as a standard (Green et al. 1982). In cartilage experiments, tissue pieces were weighed and results were expressed as pmol of nitrite / mg of tissue. In some experiments, the nitrite measurements by Griess method were confirmed by the ozone-chemiluminescence method (Braman and Hendrix 1989), with very similar results (data not shown).

3 Western blot analysis

Frozen cartilage specimen milled with Micro-Dismembrator and cell pellets from J774 cells or H4 chondrocytes were lysed in extraction buffer (10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodium orthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate, 10 µM n-octyl-β-D-glucopyranoside). Following incubation on ice for 15 min, samples were centrifuged and supernatants were mixed with sample buffer 1:4 (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 0.025% bromophenol blue and 5% β-mercaptoethanol) and stored at -20ºC until analysed. The Coomassie blue method was used to measure the protein content of the samples (Bradford 1976). After boiling, protein samples were separated with 8% SDS-polyacrylamide electrophoresis gels and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Biosciences UK limited, Buckinghamshire, UK). Proteins were identified by Western blot using rabbit polyclonal primary antibody for human or murine iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mitogen-activated protein kinases (MAPKs) were detected using specific primary antibodies for c-Jun N-terminal kinase (Jnk), p38, extracellular signal-regulated kinase 1 and 2 (Erk1/2), phospho-specific Jnk, phospho-specific p38 and phospho-specific Erk1/2 MAPK antibodies (Cell Signaling Technology Inc, Beverly, MA, USA). Bound antibody was detected using the ECL Western blot detection system (Amersham International, Buckinghamshire, UK) and then exposed to film (Kodak Biomax, Rochester, New York, USA)

(I-III) or using SuperSignal® West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and the FluorChem™ 8800 imaging system (Alpha Innotech, San Leandro, CA, USA). Quantitation of the chemiluminescent signal was carried out with FluorChem™ software v.3.1.

4 Immunohistochemistry (I)

Immunolocalisation of iNOS was carried out on cryostat sections of the cartilage tissue specimens collected during joint replacement operations in 7 patients. Cryostat sections (5-6 μm thick) were cut from different blocks from each patient. The sections were fixed in acetone/methanol fixative (50/50) at -20°C and washed with 0.05M/L tris-HCl buffered saline pH 7.6 (TBS) and immunostained with the biotin-streptavidin alkaline phosphatase technique as previously described (Moilanen et al. 1997). The sections were incubated for 18 hours with the primary antibodies that included the polyclonal sheep IgG antibody to human iNOS (1/500, a gift from Dr I. Charles), the chondrocyte marker 5B5 (1/100, a monoclonal antibody 5B5 to the β-subunit of prolyl 4-hydroxylase), and the negative control normal sheep IgG both obtained from Dako, Glostrup, Denmark. The monoclonal antibody 5B5 to the β-subunit of prolyl 4-hydroxylase, a marker of fibroblasts and chondrocytes, was purchased from Dako. This was followed by 1 h incubation with biotinylated rabbit anti sheep IgG antibody, sections stained with the 5B5 monoclonal antibody were incubated with biotinylated horse anti mouse IgG antibody. Alkaline phosphatase streptavidin conjugate was added to all slides for 1 h incubation. The substrate reaction was developed using Naphthol AS-BI phosphate and Fast Red TR salt. Levamisole was used at a concentration of 10 mM as an inhibitor of endogenous alkaline phosphatase. To confirm the specificity of the immunostaining, the primary antibody was replaced with TBS in one section. In addition, two sections from each cartilage were stained with the substrate solution with or without levamisole to exclude the possibility of nonspecific labelling of endogenous alkaline phosphatase.

5 Enzyme-linked immunosorbent assay (ELISA) (II,III)

Aliquots of the culture media were kept at -20°C until assayed. The concentrations of TNFα were determined by ELISA using reagents from Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands. sTNFRI, sTNFRII, IL-1Ra and

sIL-1RII were determined by using reagents from R&D Systems, Inc, Minneapolis, USA. (Quantikine Human sTNFRI, sTNFRII, IL-1Ra and IL-1 sRII Immunoassay). Detection limit and inter-assay coefficients of variation were 0.7 pg/ml and 7.5% for TNFα, 3.9 pg/ml and 6.7% for sTNFRI, 3.9 pg/ml and 4.1% for sTNFRII, 1.95 pg/ml and 8.7% for IL-1Ra and 3.9 pg/ml and 5.9% for sIL-RII.

6 Real-time polymerase chain reaction (Real-time PCR) (IV, V)

Chondrocyte monolayers were washed with PBS, lysed and purified with QIAshredder (QIAGEN, Valencia, CA, USA). RNA was extracted using RNeasy kit for isolation of total RNA (QIAGEN). TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA) were used to reverse-transcribe (RT) total RNA (25 ng) to cDNA with the following parameters: incubation at 25°C for 10 min, RT at 48°C for 30 min, and RT inactivation at 95°C for 5 min. After the RT reaction, cDNA (amount corresponding to approximately 1 ng of total RNA) was subjected to PCR using TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems). The primer and probe sequences were designed using Primer express® Software, purchased from Metabion, Plannegg-Martinsried, Germany and concentrations were optimized according to the manufacturer's guidelines in Taqman Universal PCR Master Mix Protocol part number 4304449 revision C and were as follows: 5'-CCTGGTACGGGCATTGCT-3', 5'-GCTCATGCGGCCTCCTT-3', (forward and reverse mouse iNOS primers, respectively, both 300 nM), 5'-CAGCAGCGGCTCCATGACTCCC -3' (mouse iNOS probe, 150 nM, containing 6-FAM [6-carboxy-fluoroscein] as 5'-reporter dye and TAMRA [6-carboxy-tetramethyl-rhodamine] as 3'-quencher), 5'-GCATGGCCTTCCGTGTTC-3', 5'-GATGTCATCATACTTGGCAGGTTT-3' (forward and reverse mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, respectively, both 300 nM), 5'-TCGTGGATCTGACGTGCCGCC-3' (mouse GAPDH probe, 150 nM, containing 6-FAM as 5'-reporter dye and TAMRA as 3'-quencher). Amplification was performed using the following protocol: incubation at 50°C for 2 min, incubation at 95°C for 10 min and thereafter 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min. Each sample was determined in duplicate.

Relative mRNA levels were determined using the standard curve method as described in Applied Biosystem User Bulletin number 2: Standard curve for both genes was created using mRNA isolated from LPS-stimulated J774 macrophages. Isolated RNA was reverse-transcribed as described. Dilution series were made from the obtained cDNA, ranging from 1 pg to 10 ng and were subjected to real-time PCR as described. The obtained threshold cycle values were plotted against the dilution factor to create a standard curve. Relative mRNA levels in test samples were then calculated from the standard curve. Results of iNOS mRNA levels were normalised against GAPDH mRNA in each sample.

7 Determination of cell viability (V)

As a measure of cell viability, cell respiration was assessed using colorimetric assay Cell Proliferation kit II (Roche Diagnostics GmbH, Mannheim Germany). Cells were seeded on 96-well plates, and incubated for 20 h at 37°C with the tested compounds. A tetrazolium salt XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate; 0.3 mg/ml), which is metabolized to formazan by intact mitochondrial dehydrogenases, and an electron coupling reagent (N-methyl dibenzopyrazine methyl sulphate; 1.25 mM) were added and cells were incubated for another 4 h. The amount of formazan accumulated into the growth medium was assessed spectrophotometrically. Triton-X treated cells were used as a positive control.

8 Electrophoretic mobility shift assay (EMSA) (V)

Cells were incubated in the presence of the tested compounds for 60 min. Thereafter monolayers were washed with PBS and solubilized in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotin, 0.1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF). After incubation for 10 min on ice, the cells were vortexed for 30 s and the nuclei separated by centrifugation at 4 °C, 21000 g for 10 s. Sample were resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotin, 0.1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF) and incubated for 20 min on ice. Samples were then vortexed for 30 s and nuclear extracts were obtained by

centrifugation at 4 °C, 21000 g for 2 min. The protein content of the nuclear extracts was measured by the Coomassie blue method (Bradford 1976).

Double-stranded transcription factor consensus oligonucleotides containing the NF-κB-binding sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3', Promega, Madison, WI, USA) were 5'[^{32}P]-end labelled (adenosine 5'-[^{32}P]triphosphate from Amersham Biosciences, Buckinghamshire, UK) with polynucleotide kinase (Roche Diagnostics GmbH, Mannheim, Germany). For binding reactions, equal amounts of nuclear extracts were incubated in 20 µl of total reaction volume containing 0.1 mg/ml (poly)dI-dC, 1 mM dithiotreitol (DTT), 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 40 mM KCl and 10% glycerol for 20 min at room temperature. Thereafter, 0.2 ng of ^{32}P-labelled oligonucleotide probe was added and the reaction mixture was incubated for 10 min at room temperature. Protein/DNA complexes were separated from free DNA probe by electrophoresis on 4% polyacrylamide gel, and the dried gel autoradiographed on film with intensifying screen at -70°C.

9 Statistics

Results are expressed as mean +/- standard error of the mean (SEM). Statistical significance of the results was calculated by Student´s t-test or by analysis of variance (ANOVA) followed by Bonferroni or Dunnett multiple comparisons test. Repeated measures ANOVA or paired t-test was used if data was matched. Differences were considered significant when $p < 0.05$ unless stated otherwise.

10 Reagents

IL-1β was purchased from Genzyme, Cambridge, MA, USA (I-III) or from R&D Systems Inc, Minneapolis, MA, USA (IV-V) and TNFα from Immugenex Corp, Los Angeles, CA, USA. IL-17, anti-human sTNFRI and sTNFRII neutralizing antibodies, mouse IgG_1 isotype control, recombinant human IL-1Ra, anti-human IL-1Ra neutralizing antibody, and TGFβ 1, TGFβ 2 and TGFβ 3 were obtained from R&D Systems Inc, Minneapolis, MA, USA.

L-NMMA (N-monomethyl-L-arginine, NOS inhibitor) was obtained from Clinalfa, Läufelfingen, Switzerland. 1400W (N-[3-(aminomethyl)benzyl]acetamidine) and L-NIO (L-N-iminoethyl-ornithine, NOS inhibitor) were kindly given by Dr Richard Knowles,

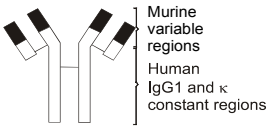
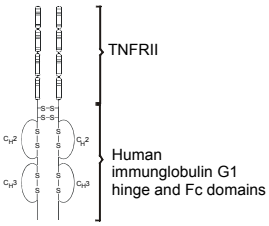
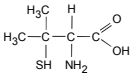
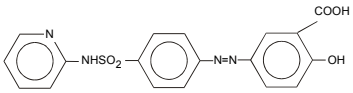
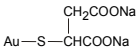
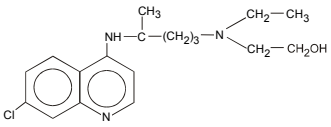
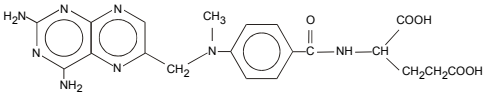
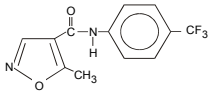
GlaxoSmithKline, Stevenage, UK. The structure and selectivity of NOS inhibitors used in the present study are presented in Table 6, on page 33.

PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one, Erk1/2 inhibitor), SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazol, p38 inhibitor) and SP600125 (anthra(1,9-cd)pyrazol-6(2H)-one, Jnk inhibitor) were obtained from Calbiochem, San Diego, CA, USA. PDTC (pyrrolidine dithiocarbamate, NF-κB inhibitor) and DAHP (diaminohydroxypyrimidine, inhibitor of BH_4 synthesis) were purchased from Tocris, Langford, Bristol, UK.

Dexamethasone, hydroxychloroquine and methotrexate were from Orion Corp, Espoo, Finland, etanercept from Wyeth Lederle, Finland, infliximab from Schering-Plough, Finland, leflunomide from Alexis Corporation, Lausen, Switzerland and D-penicillamine from Fluka, Buchs, Switzerland. The structure and molecular weight of disease-modifying antirheumatic drugs (DMARDs) used in the present study are presented in Table 7.

All other reagents were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Table 7. *Structure and molecular weight (MW) of disease-modifying antirheumatic drugs (DMARDs) used in the present study.*

DMARD	Structure	MW
infliximab		149.1 kDa
etanercept		150 kDa
D-penicillamine		149.2 g/mol
sulfasalazine		398.4 g/mol
aurothiomalate		390.1 g/mol
hydroxychloroquine		335.9 g/mol
methotrexate		454.5 g/mol
leflunomide		270.2 g/mol

Summary of the results

1 Spontaneous nitric oxide production in osteoarthritic cartilage through inducible nitric oxide synthase pathway (I)

In the absence of exogenous cytokines, OA cartilage explants produced low concentrations of nitrite, which is a stable end product of NO in aqueous solutions (Laurent et al. 1996) (Figure 9A). Nitrite accumulation was linear up to 72 h follow-up and it was partly inhibitable with the iNOS inhibitor L-NIO, suggesting that NO was produced through the iNOS pathway in OA cartilage.

Accordingly, human iNOS expression was shown in OA cartilage samples from patients undergoing knee replacement surgery by Western blot analysis (Figure 9B) and by immunohistochemical staining (Figure 9C). Western blot analysis was carried out with an antibody against human iNOS, which reacts with mouse, rat and human iNOS but not with neuronal or endothelial NOS. In cartilage pieces incubated without exogenous cytokines, a clear immunoreactive band was seen in Western blot analysis, and it was of similar MW as iNOS in LPS-treated J774 murine macrophages (Figure 9B). In immunohistochemical staining, a purified sheep antibody was used to detect iNOS. All cartilage sections from 7 OA patients showed high intensity of immunoreactivity with more than 70% of chondrocytes staining positive within each section (Figure 9C). The majority of the chondrocytes within the superficial outer surface of the cartilage were positive. In the deeper layers, the staining was less uniform, and was seen in chondrocytes within the clusters of the adjacent lacunae or doublets. The staining was mostly restricted to perinuclear cytoplasm.

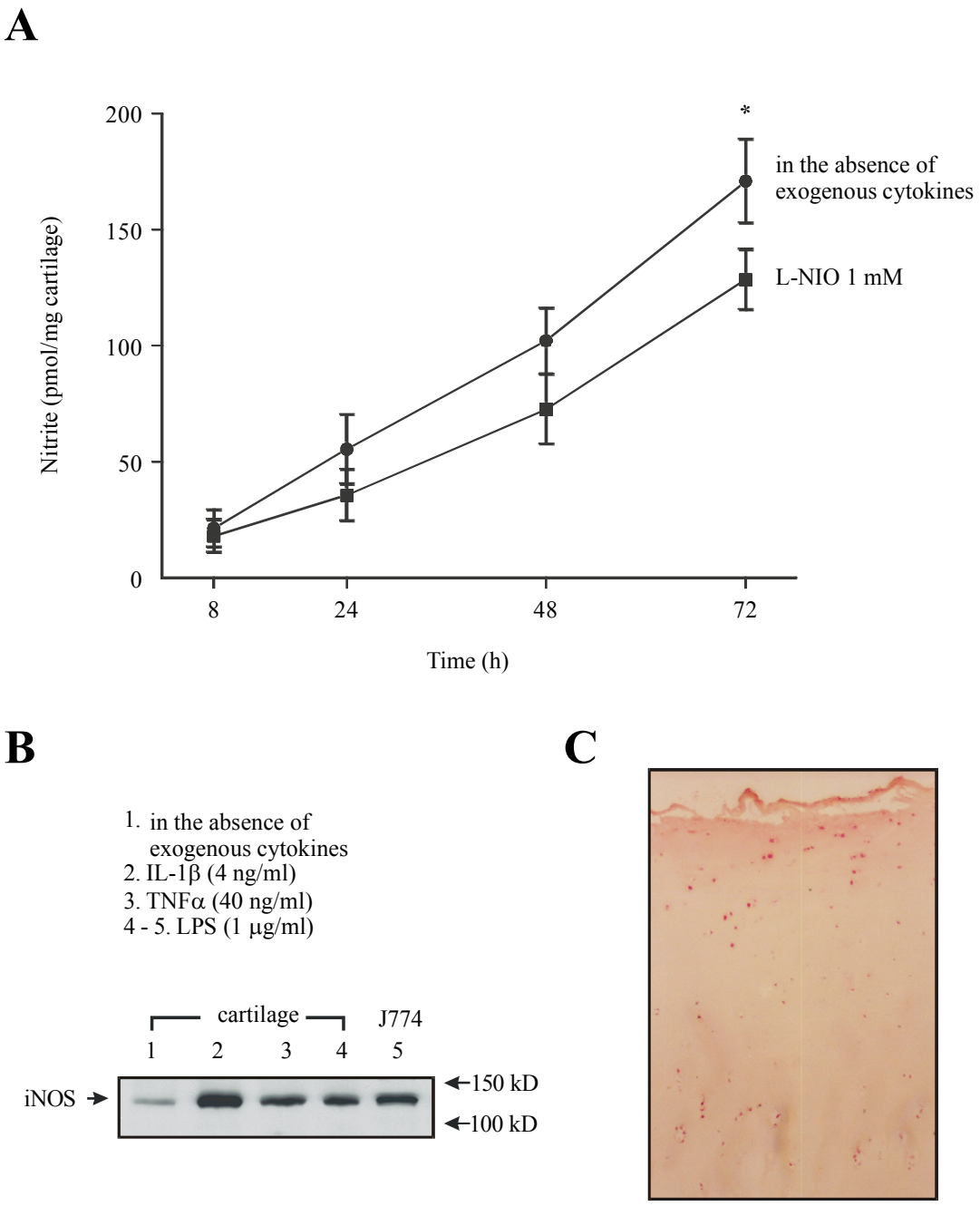


Figure 9. *NO production in OA cartilage through the iNOS pathway. (A) NO production by osteoarthritic cartilage in the absence of exogenous cytokines and the inhibitory effect of L-NIO (1 mM). Nitrite was measured in the culture medium as an indicator of NO production by Griess reaction. Results are expressed as mean ± SEM, samples were collected from 7 OA patients (n = 7), and experiments were run with 6 parallels. Statistical significance was calculated with repeated measures ANOVA followed by the Bonferroni multiple comparisons test. * = $p < 0.05$ as compared to cartilage explants treated with L-NIO. (B) iNOS expression in OA cartilage analysed by Western blot. Osteoarthritic cartilage samples were incubated for 48 h in the absence of exogenous cytokines (lane 1), with IL-1β (4 ng/ml; lane 2), TNFα (40 ng/ml; lane 3) or bacterial endotoxin LPS (1 µg/ml; lane 4). J774 macrophages stimulated with LPS for 12 h (lane 5) were used as a positive control. A representative gel is shown, one of 3 experiments with similar results. (C) Immunohistochemical section showing positive staining for iNOS in osteoarthritic cartilage, a representative section is shown from 7 OA patients - the others exhibited similar results. (Reprinted with permission from: Vuolteenaho et al. 2001, Osteoarthritis Cartilage, 9: 597-605. © Elsevier Ltd.)*

2 Regulation of nitric oxide production in osteoarthritic cartilage and in chondrocytes (I, IV)

Proinflammatory cytokines IL-1β (0.04 - 4 ng/ml) and TNFα (0.4 - 40 ng/ml) and bacterial endotoxin LPS (10 - 1000 ng/ml) stimulated NO production in OA cartilage explants in a dose-dependent manner. NO production in response to each of these stimuli was linear up to 72 h follow-up. A selective iNOS inhibitor L-NIO prevented IL-1β-, TNFα- and LPS -induced NO accumulation. Moreover, the IL-1β -induced NO production in OA cartilage explants was inhibitable with various NOS inhibitors in a dose-dependent manner in the following order of potency: 1400W (a highly selective iNOS inhibitor) = L-NIO (an iNOS inhibitor) > L-NMMA (a non-selective NOS inhibitor), whereas L-NAME (a rather selective inhibitor of the neuronal type of NOS) was quite inefficient (Figure 10). The protein synthesis inhibitor, cycloheximide, suppressed cytokine-induced NO production by 90%, indicating that it was dependent on *de novo* protein synthesis. At the protein level, Western blot analysis with human iNOS antibody showed enhanced iNOS expression in samples treated with the proinflammatory cytokines, IL-1β and TNFα, and bacterial endotoxin LPS (Figure 9B). The data indicate that in these conditions, NO production in human OA cartilage is derived from *de novo* produced iNOS.

The murine H4 chondrocyte cell line derived from mature articular cartilage was used in some experiments. Induction of NO synthesis and the signaling mechanisms involved in IL-1β -induced NO production were studied and compared to those found in OA cartilage and J774 macrophages. IL-1β (IL-1β; 10 - 1000 pg/ml) stimulated NO production in murine H4 chondrocytes in a dose- and time-dependent (Figure 11A) manner, and IL-1β -induced NO production was inhibitable with the highly selective iNOS inhibitor 1400W. Western blot analysis with an iNOS antibody showed IL-1β -induced iNOS expression (Figure 11B) and real-time PCR detected transient iNOS mRNA expression, which peaked at 4 hours after addition of IL-1 β (Figure 11C).

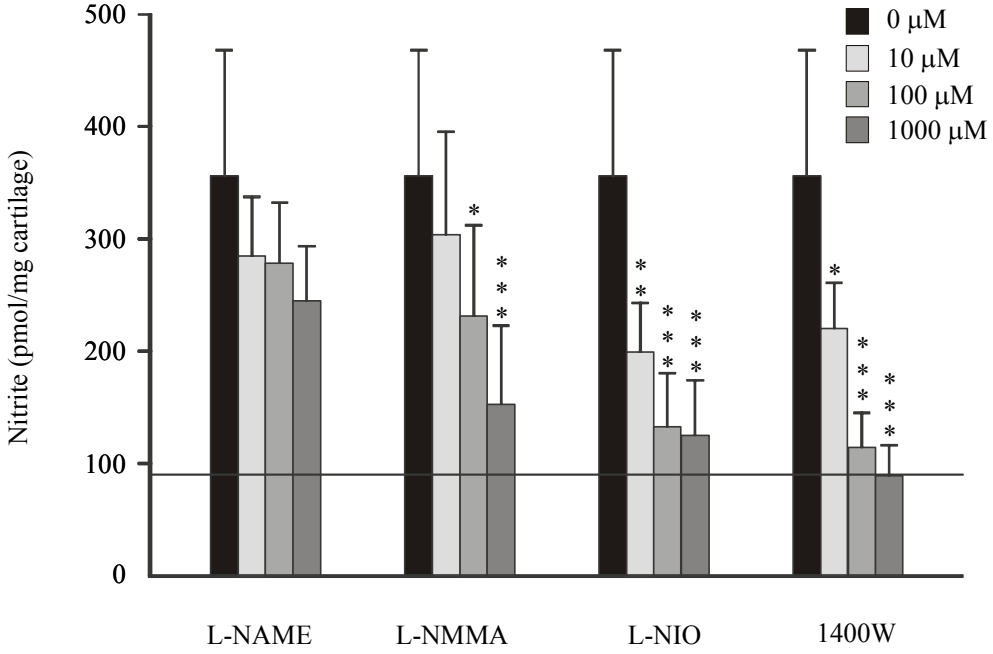


Figure 10. *The inhibition of IL-1β (4 ng/ml) -induced NO production in OA cartilage by various NOS inhibitors. NOS inhibitors were added into the culture medium at the beginning of the 42 h incubation. The horizontal line shows nitrite concentration produced by OA cartilage in the absence of exogenous IL-1β. Nitrite was measured in the culture medium as an indicator of NO synthesis by Griess reaction. Results are expressed as mean ± SEM, samples were collected from 5 patients (n=5), and experiments were run with 4 parallels. Statistical significance was calculated with repeated measures ANOVA. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 as compared to control samples incubated in the absence of NOS inhibitors. (Reprinted with permission from: Vuolteenaho et al. 2001, Osteoarthritis Cartilage, 9: 597-605. © Elsevier Ltd.)*

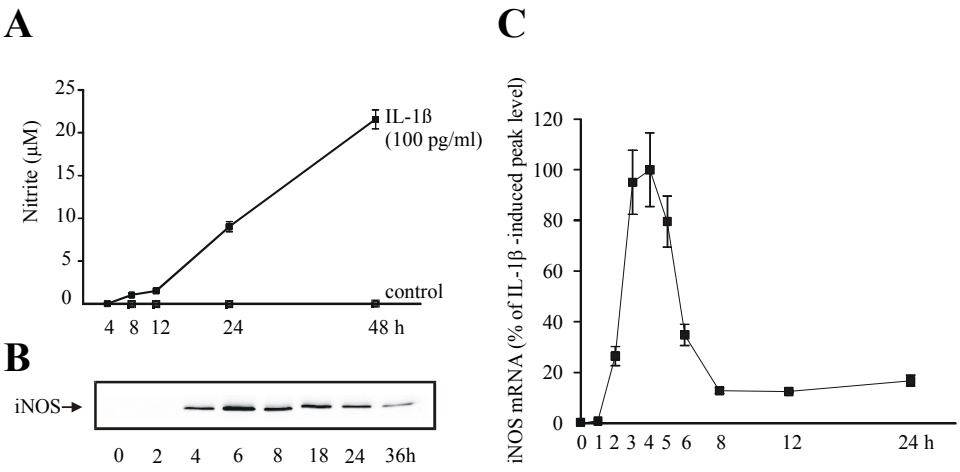


Figure 11. *The effect of IL-1β on NO production and iNOS expression in murine H4 chondrocytes. (A) Chondrocytes were incubated with IL-1β (100 pg/ml) and incubations were terminated at indicated time points. Nitrite was measured in the culture medium as an indicator of NO synthesis by Griess reaction. Results are expressed as mean ± SEM, n = 4. (B and C) Time-dependent iNOS expression in IL-1β -treated (100 pg/ml) chondrocytes analysed by (B) Western blot and (C) real-time PCR. (B) A representative gel is shown, one of 3 experiments with similar results. (C) Total RNA was extracted at the indicated time-points and iNOS mRNA was measured by real-time PCR. The results were normalized against GAPDH. Results are expressed as mean ± SEM, n = 3. (Reprinted with permission from: Vuolteenaho et al. 2005, Inflamm Res, in press. © Birkhäuser Basel)*

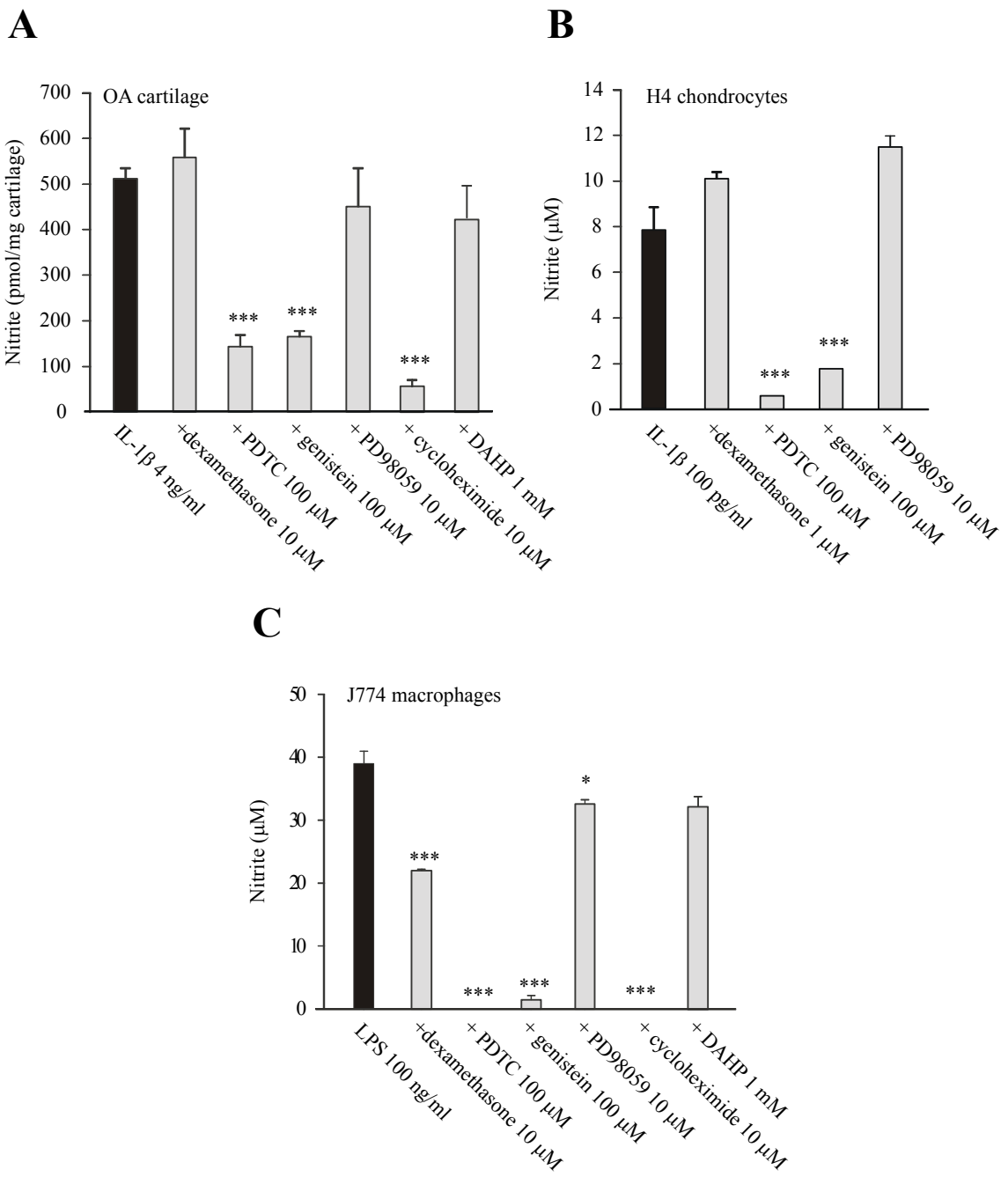


Figure 12. *The effects of dexamethasone (anti-inflammatory steroid), PDTC (ammonium pyrrolidinedithiocarbamate; inhibitor of NF-κB), genistein (tyrosine kinase inhibitor), PD98059 (inhibitor of Erk1/2 MAP-kinase), cycloheximide (inhibitor of protein synthesis) and DAHP (diaminohydroxypyrimidine, inhibitor of BH_4 synthesis) on NO production in (A) osteoarthritic cartilage stimulated with IL-1β (4 ng/ml) for 42 h, in (B) H4 chondrocytes stimulated with IL-1β (100 pg/ml) for 24 h and in (C) J774 macrophages stimulated with LPS (100 ng/ml) for 24 h. The tested compound was added into the culture medium at the beginning of the incubation. Nitrite was measured in the culture medium as an indicator of NO production by Griess reaction. Results are expressed as mean ± SEM, (A) samples were collected from 6 OA patients (n = 6), and experiments were run with 4 parallels, (B) n=4 and (C) n=6. Statistical significance was calculated with repeated measures (A) or ordinary (B and C) ANOVA followed by the Bonferroni multiple comparisons test. *** = $p < 0.001$, * = $p < 0.05$ as compared to control samples incubated in the absence of pharmacological agents. (Reprinted with permission from: Vuolteenaho et al. 2001, Osteoarthritis Cartilage, 9: 597-605. © Elsevier Ltd.)*

Signaling mechanisms involved in the IL-1 -induced NO production in OA cartilage, in H4 chondrocytes and for comparison in LPS -stimulated J774 macrophages were studied by pharmacological means. PDTC (ammonium pyrrolidinedithiocarbamate, an inhibitor of NF-κB) and genistein (an inhibitor of tyrosine protein kinase) significantly inhibited IL-1β -induced NO production in OA cartilage and in murine H4 chondrocytes, and LPS -induced NO production in J774 macrophages, while the inhibitor of MAP-kinase Erk1/2 (PD98059) had only a minor effect in J774 cells and the inhibitor of tetrahydrobiopterin (BH_4) synthesis (DAHP, diaminohydroxypyrimidine) had no effect. Dexamethasone suppressed LPS -induced NO production in J774 macrophages by 40%, while there was no effect on IL-1β -induced NO synthesis in osteoarthritic cartilage and in H4 chondrocytes (Figure 12). The results suggest that IL-1β -induced NO synthesis in osteoarthritic cartilage and H4 chondrocytes and LPS -induced NO production in murine macrophages is dependent on NF-κB activation and tyrosine kinases, while Erk1/2 MAP-kinase and BH_4 synthesis seem to have no role in the process. In the respect of studied mechanisms, NO production in human OA cartilage and H4 chondrocytes derives from glucocorticoid-insensitive expression of iNOS and very similar mechanisms seem to regulate NO production in J774 macrophages with the exception of dexamethasone-sensitivity.

3 Endogenous cytokine antagonists regulate nitric oxide production in human cartilage (II, III)

OA cartilage responded to IL-1β -stimulation with higher NO production than RA cartilage, whereas there was no significant difference in NO synthesis between OA and RA samples in their response to TNFα and LPS. The ability of human cartilage to regulate response to IL-1β -treatment by producing endogenous IL-1 antagonists was studied by measuring the concentrations of IL-1 receptor antagonist (IL-1Ra) and soluble IL-1 receptor II (sIL-1RII) released from the cartilage explants into the culture medium by ELISA in the absence of exogenous cytokines. RA cartilage produced higher amounts of both IL-1Ra (Figure 13A) and sIL-1RII than OA cartilage. sIL-1RII concentrations measured in the culture medium were 78.4 ± 67.7 pg/100 mg RA cartilage (n=4) vs. 13.0 ± 5.2 pg/100 mg OA cartilage (n=6).

To confirm the functional ability of IL-1Ra to suppress IL-1β -induced NO synthesis in OA cartilage, the effect of exogenous IL-1Ra was studied. IL-1β (4 ng/ml) induced NO production

up to 430.7 ± 68.0 pmol/mg cartilage and IL-1Ra 500 ng/ml reduced IL-1β -induced NO production down to 210.6 ± 26.7 pmol/mg cartilage ($p<0.01$).

Spontaneous IL-1Ra production (1.26 ± 0.63 pg/mg cartilage) in OA cartilage was enhanced to 3.82 ± 0.73 pg/mg cartilage ($p<0.05$) after treatment with IL-1β (Figure 13B). A highly selective iNOS inhibitor 1400W further enhanced IL-1β -induced IL-1Ra production up to 6.81 ± 0.85, i.e. it nearly doubled the amount produced in the presence of IL-1β alone. In these conditions, 1400W nearly totally (> 95%) inhibited IL-1β -induced NO production in OA cartilage, suggesting that NO down-regulates IL-1Ra production.

In addition to IL-1 antagonists, OA cartilage released endogenous TNFα antagonists, soluble TNF receptor I and II (sTNFRI; 215.0 ± 18.3 pg/100 mg cartilage and II; 605.7 ± 184.6 pg/100 mg cartilage), into the culture medium as measured by ELISA. A neutralizing antibody against soluble TNFRI enhanced NO production by 124%, while antibody against soluble TNFRII or control IgG were ineffective (Figure 14). The result suggests that an endogenous cartilage-derived TNFα antagonist modulates NO production in OA cartilage.

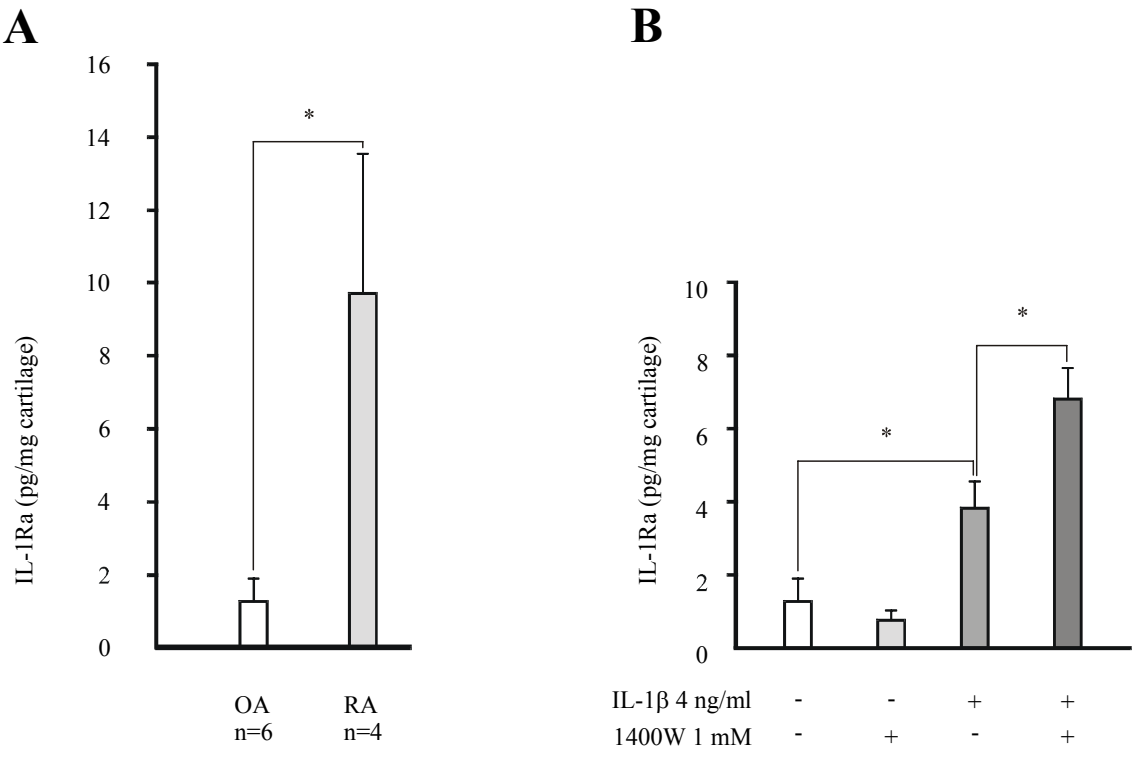


Figure 13. *IL-1Ra released from cartilage. (A) OA and RA cartilage explants were incubated in the absence of exogenous cytokines for 42 h. (B) The effect of a highly selective iNOS inhibitor 1400W (1 mM) on IL-1Ra production in IL-1β (4 ng/ml) -stimulated OA cartilage during 42 h incubation. (A and B) IL-1Ra was measured in the culture medium by ELISA and expressed as pg/mg cartilage. Statistical significance was calculated with (A) unpaired and (B) paired t-test. Mean ± SEM, samples were collected from 6 OA patients (n = 6) and 4 RA patients (n=4) and experiments were run with 4 parallels. * = $p < 0.05$ as compared to indicated control. (Reprinted with permission from: Vuolteenaho et al. 2003, Scand J Rheumatol, 32: 19-24. © Taylor & Francis)*

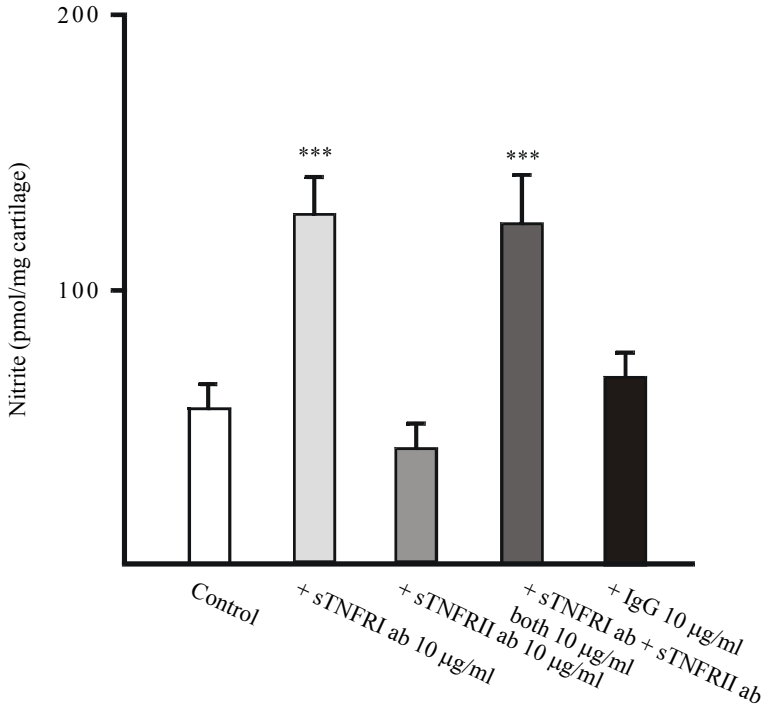
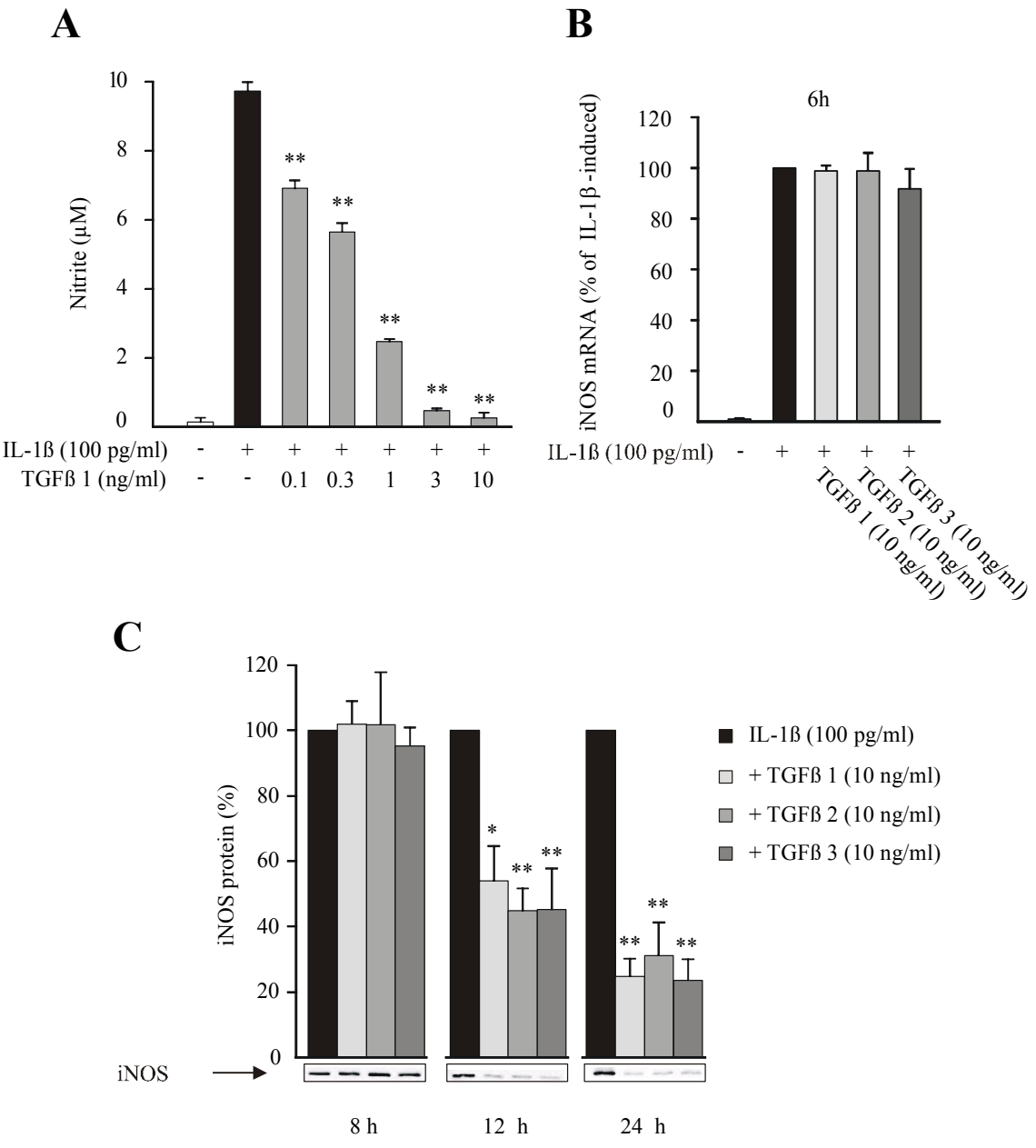


Figure 14. *The effects of neutralizing antibodies against soluble TNF receptors I (sTNFRI ab; 10 µg/ml) and II (sTNFRII ab; 10 µg/ml) on NO production in OA cartilage during 42 h incubation. IgG (10 µg/ml) was used as a negative control. Results are expressed as mean ± SEM, samples were collected from 6 OA patients (n = 6), and experiments were run with 3 parallels. Statistical significance was calculated with repeated measures ANOVA followed by the Bonferroni multiple comparisons test.* *** = $p < 0.001$ *as compared to control without sTNFR antibodies. (Reprinted with permission from: Vuolteenaho et al. 2002, Osteoarthritis Cartilage, 10: 327-332. © Elsevier Ltd.)*

4 Transforming growth factor β inhibits nitric oxide production by enhancing the degradation of inducible nitric oxide synthase protein in chondrocytes (IV)

TGFβ is regarded mainly as an anabolic factor in OA cartilage that counteracts the destructive effects of IL-1 and TNFα. The effect of TGFβ was investigated on IL-1β -induced NO production in H4 chondrocytes. TGFβ (TGFβ 1, TGFβ 2 and TGFβ 3; 0.1 - 10 ng/ml) inhibited IL-1β -induced NO production in H4 chondrocytes in a concentration-dependent manner (Figure 15A). Interestingly, TGFβ (TGFβ 1, TGFβ 2 and TGFβ 3) had no effect on iNOS mRNA levels, when measured 3 h or 6 h after addition of IL-1β (Figure 15B). iNOS protein levels were similar in cells treated with IL-1β or IL-1β + TGFβ when measured after 8 h incubation, whereas when measured after 12 h and 24 h incubations, iNOS protein levels were 50% and 80% lower in cells treated with IL-1β + TGFβ than in cells treated with IL-1β alone

(Figure 15C). To test the effect of TGFβ on iNOS protein stability cycloheximide, an inhibitor of protein synthesis, was added into the cell culture 8 hours after induction of iNOS protein expression by cytokines. Proteins were extracted 4, 8, 16 and 28 hours after cycloheximide treatment. iNOS protein levels halved in 8 h in IL-1β -treated cells and in 4 h in cells treated with the combination of IL-1β and TGFβ 1 (Figure 15D). The results suggest that TGFβ enhances the degradation of iNOS protein in chondrocytes exposed to IL-1β.



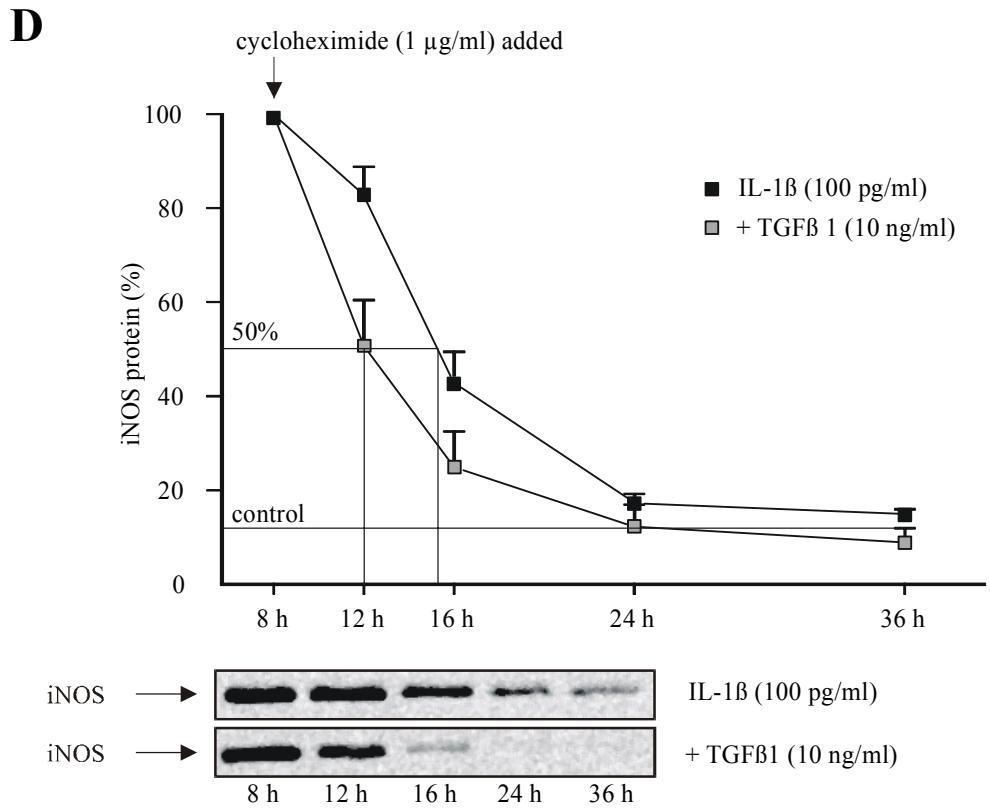


Figure 15. *The effect of TGFβ on IL-1β -induced NO production, and iNOS mRNA and protein expression in H4 chondrocytes. (A) Chondrocytes were incubated with IL-1β (100 pg/ml) and increasing concentrations of TGFβ 1 (0.1 - 10 ng/ml) for 48 h. Nitrite was measured in the culture medium as an indicator of NO synthesis by Griess reaction. (B) Chondrocytes were incubated with IL-1β (100 pg/ml) and TGFβ 1, TGFβ 2 or TGFβ 3 (10 ng/ml) for 6 h. Total RNA was extracted at the indicated time points and iNOS mRNA was measured by real-time PCR. The results were normalized against GAPDH. (C) Chondrocytes were incubated with IL-1β (100 pg/ml) and TGFβ 1, TGFβ 2 or TGFβ 3 (10 ng/ml). Incubations were terminated at the indicated time points and iNOS protein was measured by Western blot. At each time point, iNOS protein level in IL-1β -treated cells was set as 100% and iNOS in IL-1β + TGFβ -treated cells was expressed as a percentage of that value. (D) Cells were incubated with IL-β (100 pg/ml) with or without TGFβ 1 (10 ng/ml). After 8 h incubation cycloheximide (1 µg/ml), an inhibitor of protein synthesis, was added into the culture medium. Proteins were extracted after the indicated time points and iNOS was analysed by Western blot. The control line shows level of iNOS expression in the absence of exogenous cytokines at 8 h. (A, B, C and D) Results are expressed as mean ± SEM, n = 3. Statistical significance was calculated with ordinary ANOVA followed by the Dunnet multiple comparisons test. * = $p < 0.05$ and ** = $p < 0.01$ as compared to chondrocytes treated with IL-1β alone. (Reprinted with permission from: Vuolteenaho et al. 2005, Inflamm Res, in press. © Birkhäuser Basel)*

5 The effect of disease-modifying antirheumatic drugs on nitric oxide synthesis in chondrocytes and in osteoarthritic cartilage (II, V)

The effect of TNFα -antagonists, infliximab and etanercept, was studied on NO production in OA cartilage. TNFα -induced NO production and iNOS expression was suppressed by infliximab (0.1 - 100 μg/ml) and etanercept (1 - 1000 ng/ml) in a concentration-dependent manner. In contrast, spontaneous NO production or that induced by IL-1β, IL-17 or LPS was not altered by infliximab or etanercept. This finding suggests that TNFα is not an autacoid-mediator in these processes.

In addition to the TNFα -antagonists, the effect of six other DMARDs were studied in chondrocytes and in OA cartilage. Aurothiomalate (10 - 50 μM), hydroxychloroquine (25 - 100 μM), methotrexate (1 - 100 μM) and leflunomide (50 - 100 μM), inhibited IL-1β -induced NO production in H4 chondrocytes, while penicillamine and sulfasalazine had no effect when used at concentrations up to 100 μM. In the subsequent studies, drugs were used in concentrations that caused their maximal inhibitory effect (Table 8). Treatment with aurothiomalate, hydroxychloroquine, methotrexate and leflunomide suppressed IL-1β -induced activation of NF-κB, which is a critical transcription factor for iNOS (Figure 16A). Subsequently, iNOS mRNA (Figure 16B) and protein (Figure 16C) expression were also inhibited. The four effective DMARDs were tested also in OA cartilage. Aurothiomalate and hydroxychloroquine inhibited IL-1β -induced NO production in OA cartilage whereas methotrexate and leflunomide had no effect (Figure 16D).

Table 8. *Dose-dependent effects of DMARDs on IL-1β (100 pg/ml) -induced NO production in H4 chondrocytes.*

Compound	**Concentration (μM)**	**Nitrite (% of IL-1β -induced)**
IL-1β 100 pg/ml		100
+Aurothiomalate	10	43.2 ± 3.1 (**)
	25	6.4 ± 1.8 (**)
	50	6.2 ± 1.7 (**)
+Hydroxychloroquine	25	80.2 ± 4.5 (**)
	50	55.8 ± 5.8 (**)
	75	35.0 ± 4.7 (**)
	100	25.8 ± 6.4 (**)
+Methotrexate	1	79.6 ± 3.1 (**)
	3	70.0 ± 1.4 (**)
	10	56.1 ± 2.9 (**)
	100	61.9 ± 5.3 (**)
+Leflunomide	50	87.4 ± 14.7
	75	72.8 ± 14.9
	100	52.8 ± 7.2 (*)

*Cells were incubated for 24 h with the tested DMARD and IL-1β. Nitrite was measured in the culture medium as an indicator of NO production by Griess reaction. Results are expressed as mean ± SEM, n = 6. Statistical significance was calculated with ordinary ANOVA followed by the Dunnet multiple comparisons test. * = $p < 0.05$ and ** = $p < 0.01$ as compared to chondrocytes treated with IL-1β only. The underlined drug concentration was used in the further experiments. (Reprinted with permission from: Vuolteenaho et al. 2005, Scand J Rheumatol, in press. © Taylor & Francis)*

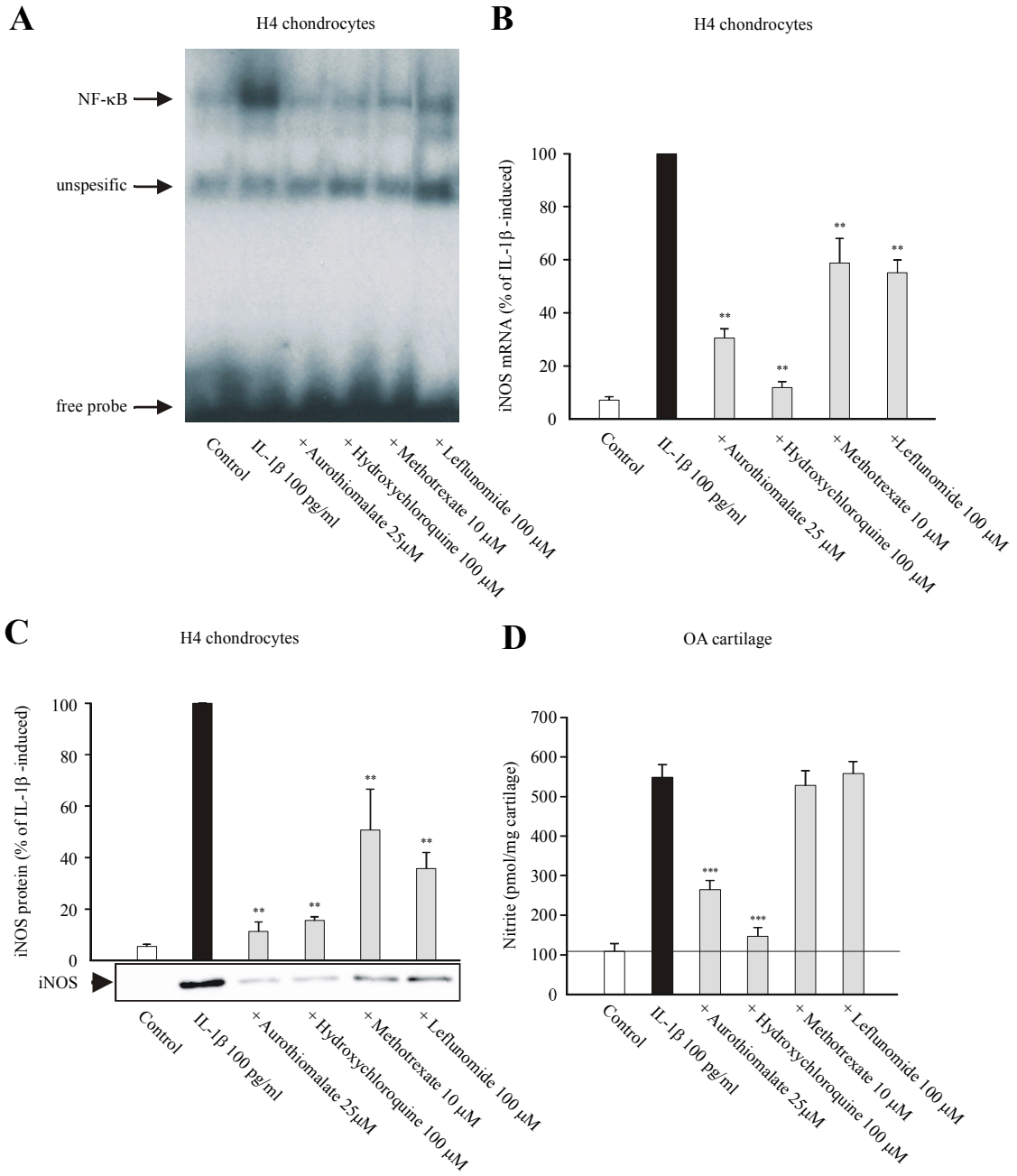


Figure 16. *The effects of DMARDs on IL-1β (100 pg/ml) -induced (A) NF-κB activation, (B) iNOS mRNA and (C) iNOS protein expression in H4 chondrocytes and (D) NO production in OA cartilage. (A) Cells were incubated with the tested DMARD and IL-1β for 60 min. NF-κB activation was determined by EMSA. A representative gel is shown, one of 3 experiments with similar results. (B) Chondrocytes were incubated with the tested DMARD and IL-1β for 2 h. Total RNA was extracted at the indicated time points and iNOS mRNA was measured by real-time PCR. The results were normalized against GAPDH mRNA. Results are expressed as mean ± SEM, n = 3. (C) Cells were incubated for 24 h with the tested DMARD and IL-1β. iNOS protein was measured using Western blot. Results are expressed as mean ± SEM, n = 3. (B and C) Statistical significance was calculated with ordinary ANOVA followed by the Dunnet multiple comparisons test. * = $p < 0.05$ and ** = $p < 0.01$ as compared to chondrocytes treated with IL-1β only. (D) Cartilage explants were incubated for 48 h with the tested DMARD and IL-1β. Nitrite was measured in the culture medium as an indicator of NO production by Griess reaction. Results are expressed as mean ± SEM, samples were collected from 6 OA patients (n = 6), and experiments were run with 4 parallels. *** = $p < 0.001$ as compared to cartilage explants treated with IL-1β only. (Reprinted with permission from: Vuolteenaho et al. 2005, Scand J Rheumatol, in press. © Taylor & Francis)*

Discussion

1 Methodology

As a reactive molecule, NO is readily oxidized to nitrite and nitrate in aqueous and biological solutions, making direct measurement of NO production difficult. In the presence of oxyhaemoglobin e.g. in blood, NO is predominantly oxidized to nitrate, but in cell culture conditions nitrite is the main metabolite and this form can be used to estimate NO production (Ignarro et al. 1993, Moshage et al. 1995, Laurent et al. 1996). In the present study, the colorimetric reaction of nitrite with Griess reagent was quantificated by spectrophotometer and used to measure nitrite (Green et al. 1982). The more specific and sensitive ozonechemiluminescence method (Braman and Hendrix 1989) was used to confirm the suitability of Griess reaction to measure nitrite under these conditions and to exclude interference of medium constituents and cartilage products with Griess reagent. NOS inhibitors were used to differentiate nitrite derived from other biochemical pathways and cellular sources.

Standard methods in molecular and cellular biology were used to detect iNOS protein (Western blot), iNOS mRNA (real-time PCR), NF-κB (EMSA) and cytokines (ELISA). Protein degradation was estimated by measuring protein levels at different intervals after addition of the protein synthesis inhibitor, cycloheximide. Inhibition of protein synthesis allows evaluation of protein half-life, but may interfere with protein degradation, if this is dependent on *de novo* protein synthesis.

Articular cartilage degradation is the main feature of OA pathology and cartilage explants and the only cell type present in the cartilage, i.e. chondrocytes, were used in study. Use of cartilage explants in tissue culture has advantages as compared to chondrocyte monolayer cell culture, although in both methods, the incubator atmosphere with 95% air/5% CO_2 (v/v), 37°C temperature and culture medium are not identical to the ascetic environmental conditions surrounding the chondrocyte within the joint. In the cartilage explants, chondrocytes live in their natural microenvironment within the matrix, allowing chondrocytes to interact with matrix components in three-dimensional surroundings, where chondrocytes are sparsely dispersed. These conditions maintain the characteristic chondrocyte phenotype: i.e. an infrequently dividing cell, which secretes cartilage-specific keratan-sulphate and type II collagen and has a

round appearance. Similar to the *in vivo* situation, high molecular size or fixed charge of tested compound may prevent penetration into the avascular cartilage matrix (Evans et al. 1996, Schleyerbach and Raiss 2002).

The cartilage tissue used in this study was obtained from the pieces leftover from total knee replacement surgery. A cartilage specimen from the early stages of OA would be more relevant to study, but there is very limited availability of such human tissue. However, OA knee with typical varus-alignment shows the most severe degenerative changes of the articular cartilage in the medial compartment of the knee. Cartilage on the contralateral side of the joint seems to be macroscopically almost intact with cartilage changes related to early OA and this tissue was used in the present study. Patients' medication (for the treatment of osteoarthritis or other conditions) or anesthesia may represent confounding factors, which could not be standardized in the present study. The reproducibility of results from heterogeneous tissue samples was improved by weighing the cartilage explants after the experiments and expressing the results per mg of cartilage, by dissecting full-thickness pieces of cartilage to avoid a cartilage layer effect, and by collecting samples from at least 4 donor patients, each experiment being run with 4-6 parallels. The limited amount of tissue restricts the size of experiments and number of compounds tested (Evans et al. 1996, Schleyerbach and Raiss 2002). Low cellularity, high concentrations of proteoglycan aggrecans and their electronegativity impairs isolation of mRNA from the cartilage (Chomczynski and Sacchi 1987, Adams et al. 1992, Re et al. 1995).

To overcome the limitations of cartilage explant experimentation for the present study, an immortalized chondrocyte cell-line was searched. Immortalization of chondrocytes often results in loss of chondrocyte phenotype and successful human chondrocyte cell-lines have not been available until T/C-28a2, T/C-28a4, C-28/I2, which originated from juvenile costal chondrocytes and tsT/AC62, which originated from adult articular cartilage were developed by Goldring et al (Goldring et al. 1994, Robbins et al. 2000, Finger et al. 2003). As a research collaboration, all but one (T/C-28a4) of these cell-lines were cultured and studied as a part of the the present study. Unfortunately, NO production in these cells was not inducible by treatments that induced NO production in OA cartilage explants and thus these cell-lines could not be used in the subsequent studies as a model to study the regulation of NO production in human OA cartilage.

The immortalized murine H4 chondrocyte cell line derived from mature femoral head articular cartilage developed and characterized by Dr van Beuningen and Prof van der Kraan was obtained for the present study. H4 chondrocytes were immortalized by transfection of the SV40 large T antigen gene and this was reported to stabilize the phenotype so that it remains comparable to differentiated chondrocytes. H4 chondrocytes have been shown to produce type II collagen and in response to IL-1 to suppress proteoglycan synthesis in alginate bead culture.

H4 chondrocytes grown in monolayer cultures have exhibited responses similar to those found in articular cartilage *ex vivo* after exposure to IL-1 and TGFβ (van Beuningen et al. 2002, Scharstuhl et al. 2003, Takahashi et al. 2005). NO synthesis in H4 cell line was not impaired in monolayer culture up to 20 passages and thus that cell line was selected for some of the studies for practical reasons.

The aim of the present study was to investigate the regulation of iNOS expression and NO production in human osteoarthritic cartilage and in H4 chondrocyte cultures. IL-1 plays an important role in the cartilage destruction in OA and therefore IL-1 was used in the present study to activate chondrocytes and induce NO production (Evans et al. 1996, Schleyerbach and Raiss 2002).

2 Nitric oxide production and inducible nitric oxide synthase expression in osteoarthritic cartilage

Increased concentrations of nitrite have been found in synovial fluid from OA patients (Farrell et al. 1992, Renoux et al. 1996, Karan et al. 2003) indicative of local NO production in the joint. Amin et al. (Amin et al. 1995) reported spontaneous nitrite release from OA cartilage in serum-free medium, which could be inhibited with a non-selective NOS inhibitor L-NMMA. Similarly we found that OA cartilage explants produced low concentrations of nitrite in the absence of exogenous cytokines in a time dependent manner. Part of this nitrite accumulation was inhibitable with the iNOS inhibitor, L-NIO, suggesting that it was derived from NO produced through iNOS pathway. However, some amounts of L-NIO -insensitive nitrite accumulation remained, even though the iNOS inhibitor was used at high (1 mM) concentrations. The ozone-chemiluminescence method was used to confirm the nitrite assays by the Griess method. The results suggest that L-NIO -insensitive nitrite was formed by biochemical pathways other than NOS or it resulted from poor penetration of L-NIO into the cartilage.

In subsequent studies, iNOS expression in human OA cartilage was confirmed by Western blot analysis and by immunohistochemical staining. Amin et al. (Amin et al. 1995) described a special NOS in OA cartilage (OA-NOS) that was found to be different from iNOS and relatively close to nNOS in its molecular weight and antibody binding. The sequence of OA-NOS was not reported by Amin et al. and there are no further publications on OA-NOS. Therefore it is likely that the so-called OA-NOS is a modification of one of the earlier documented isoforms, either iNOS or nNOS, rather than a novel isoform of NOS. The present results support the idea that

iNOS is the principal NOS isoform responsible for spontaneous and IL-1 -induced NO production in OA cartilage. Cartilage seems to be the main source of iNOS-derived NO in the joint, this finding being supported by immunostaining results that showed high expression of iNOS in cartilage as compared to low or absent expression of iNOS in synovial membrane in biopsy samples from OA patients (Melchiorri et al. 1998).

3 Glucocorticoid-insensitive interleukin-1 -induced nitric oxide production and inducible nitric oxide synthase expression in osteoarthritic cartilage and in chondrocytes

In the present study, OA cartilage and H4 chondrocytes responded to IL-1 with enhanced iNOS expression and NO production. Non-selective and iNOS selective NOS inhibitors and cycloheximide suppressed IL-1 -induced NO production, indicating that NO was produced by *de novo* synthesized iNOS. A highly selective iNOS inhibitor, 1400W (Garvey et al. 1997), was the most potent antagonist of IL-1 -induced NO production in OA cartilage, when compared to the iNOS inhibitor, L-NIO, the nNOS inhibitor, L-NAME, and the non-selective NOS inhibitor, L-NMMA. The results indicate that 1400W is able to penetrate into cartilage, which means that this inhibitor may represent a useful tool for studies on NO production in cartilage. Similarly to OA cartilage explants and J774 macrophages, IL-1 -induced NO production in H4 chondrocytes was inhibitable with genistein and PDTC, pointing to the importance of protein tyrosine phosphorylation and activation of transcription factor NF-κB as critical mediators in IL-1 -induced NO production.

Glucocorticoids are commonly administered as intra-articular injections in the treatment of knee OA (Arroll and Goodyear-Smith 2004) and are important anti-inflammatory drugs for many inflammatory and allergic disorders. Glucocorticoids have been shown to suppress inducible NO production in various cell types, e.g. in porcine vascular smooth muscle cells (Radomski et al. 1990), in rat hepatocytes (de Vera et al. 1997) and in human epithelial cells (Kleinert et al. 1996), and different transcriptional and post-transcriptional mechanisms of action have been proposed (Gotoh et al. 1996, Kleinert et al. 1996, Kunz et al. 1996, Simmons et al. 1996, de Vera et al. 1997, Korhonen et al. 2002). In contrast, induction of iNOS seems to be resistant or only marginally responsive to the suppressive effect of glucocorticoids in some cell types, e.g. in human colon epithelial cells (Salzman et al. 1996) and in human, rabbit, rat and bovine chondrocytes (Palmer et al. 1992, Palmer et al. 1993, Amin et al. 1995, Järvinen et

al. 1995, Grabowski et al. 1996, Amin et al. 1997, Attur et al. 1998, De Gendt et al. 1998, Guerne et al. 1999, Borderie et al. 2001), although also opposite results have been reported (Shalom-Barak et al. 1998, Shirazi et al. 2001). In the present study, IL-1 -induced NO production in OA cartilage and murine H4 chondrocytes was insensitive to dexamethasone.

4 Production of endogenous cytokine antagonists in osteoarthritic cartilage

OA cartilage releases an abundance of inflammatory mediators when compared to healthy cartilage (Attur et al. 2002), and within the avascular cartilage tissue, cytokines exert many of their effects on chondrocytes in an autocrine-paracrine fashion. Proinflammatory cytokines IL-1 and TNFα play central roles in the cartilage degradation related to OA (Goldring and Goldring 2004). In the present study, the endogenous regulation of the IL-1 and TNFα -cytokine network in OA and RA cartilage and how this impacts on NO production were investigated.

OA cartilage was found to produce more NO in response to IL-1 stimulation than RA cartilage, whereas there was no difference in the response to TNFα and LPS. The results indicated that the difference between OA and RA cartilage was attributable to different responsiveness to IL-1 rather than to different cellularity of the samples. RA cartilage was shown to release higher concentrations of endogenous IL-1 antagonists IL-1Ra and sIL-1RII compared to OA cartilage, which may explain the differential response to IL-1 stimulation. Due to decreased production of IL-1Ra and sIL-RII by OA cartilage as compared to RA or healthy cartilage (Pelletier et al. 1996), OA cartilage is likely to be more vulnerable to the destructive effects of IL-1.

IL-1 has been reported to induce IL-1Ra production by human articular chondrocytes (Palmer et al. 2002) and that was confirmed in the present study. Interestingly, the highly selective iNOS inhibitor, 1400W (Garvey et al. 1997), further enhanced IL-1 -induced IL-1Ra production by OA cartilage. These data are supported by previous findings showing enhancement of induced IL-1Ra production with non-selective NOS inhibitors L-NMMA and aminoguanide in human chondrocytes and cartilage (Pelletier et al. 1996, Shirazi et al. 2001). These results point to the presence of a positive feedback loop between IL-1 and NO since IL-1 -induced NO production promotes cartilage destruction by IL-1 by suppressing the production of protective IL-1Ra. A preliminary clinical trial has shown beneficial effects with intra-articular IL-1Ra therapy in symptomatic OA patients (Goupille et al. 2003) and the ability of

NOS inhibitors to enhance the production of IL-1Ra points to a further anti-inflammatory mechanism that iNOS inhibitors may have in the treatment of OA.

In the present study, OA cartilage released TNFα and endogenous TNFα-antagonists, i.e. soluble TNFRI and TNFRII. Westacott et al. (Westacott et al. 1994) have earlier reported that there were no detectable amounts of sTNFRI or sTNFRII in supernatants from cultured OA chondrocytes. The reasons for the difference are not clear but they may be attributable to chondrocyte dedifferentiation in cell culture conditions. In the present study, neutralizing antibodies against soluble receptors were used to test the possible biological activity of the detected TNFα-antagonists on NO production in the cartilage. When the effect of soluble TNFRI was blocked, NO production was enhanced, suggesting that endogenous TNFα-antagonist sTNFRI down-regulates the effects of TNFα in OA cartilage.

The effect of a cytokine depends on local concentration of the cytokine of interest, and on the expression of their regulators and receptors. Together, these factors create a cytokine network, where a change in a single factor may result in complicated feedback variations (Goldring and Goldring 2004). Novel tools for gene expression analysis and proteomics, e.g. cDNA and antibody arrays offer opportunities to detect overall changes in the network e.g. in the IL-1 signaling cascade rather than single gene/protein of interest or to identify new mediators in the OA pathogenesis (Aigner et al. 2004, De Ceuninck et al. 2004, Takahashi et al. 2005).

5 Regulation of nitric oxide production and inducible nitric oxide synthase expression by transforming growth factor β

In the present study, TGFβ inhibited IL-1 -induced NO production and iNOS expression in chondrocytes, probably by enhancing iNOS protein degradation. TGFβ did not alter iNOS mRNA levels in IL-1 -treated chondrocytes suggesting that TGFβ had no effect on iNOS transcription or on iNOS mRNA stability. TGFβ has been shown to mediate suppression of NO production in activated murine macrophages by decreasing iNOS mRNA stability, by reducing iNOS mRNA translation and by increasing iNOS protein degradation (Vodovotz et al. 1993), but it may also suppress iNOS expression at the transcriptional level or regulate iNOS activity (Vodovotz 1997).

The results of the present study showed enhanced degradation of iNOS protein in chondrocytes following treatment with TGFβ, and this mechanism may well explain the suppressive effect of TGFβ on NO production in these activated chondrocytes. In addition to

TGFβ (Vodovotz et al. 1993), dexamethasone has been shown to inhibit NO production at the level of iNOS protein stability though other mechanisms may be involved (Kunz et al. 1996). Recently, the involvement of the proteasome pathway in the degradation of iNOS protein has been suggested since addition of proteasome inhibitors has resulted in enhanced iNOS protein levels in human colon carcinoma cells (Felley-Bosco et al. 2000), in human kidney HEK293 cells with stable iNOS expression and in cytokine-treated epithelial RT4 and murine RAW 264.7 macrophages (Musial and Eissa 2001). Furthermore, a recent study reported up-regulation of the genes involved in the ubiquitin/proteasome pathway by TGFβ in H4 chondrocytes analysed by oligonucleotide arrays surveying approximately 12000 genes (Takahashi et al. 2005). This finding may offer a mechanism, by which TGFβ targets iNOS for proteasome degradation and thus enhances iNOS protein degradation in H4 chondrocytes.

In addition to the above-mentioned effects of TGFβ on iNOS expression, we studied the possible involvement of two other mechanisms based on the existing literature, i.e. the effects of TGFβ mediated through enhanced arginase activity and decreased BH_4 synthesis. L-arginine is needed for NO synthesis as a substrate and it is also metabolized to L-ornithine and urea by arginase (Boucher et al. 1999). It was recently shown that intracellular L-arginine depletion can reduce iNOS mRNA translation by inactivating eukaryotic initiation factor2α, an important factor in the initiation of translation in eukaryotes (El-Gayar et al. 2003, Lee et al. 2003). Since TGFβ has been reported to stimulate arginase activity in macrophages (Boutard et al. 1995), we tested the effects of the arginase inhibitor L-valine and those of L-arginine supplementation on the effects of TGFβ in chondrocyte cultures. Neither of these treatments altered the effects of TGFβ on NO production (data not shown) suggesting that the effects of TGFβ were not mediated through enhanced arginase activity.

TGFβ has been also shown to suppress the production of iNOS cofactor BH_4 synthesis by downregulating the expression of guanosine triphosphate-cyclohydrolase I (GTP-CHI) that is required in the *de novo* synthesis of BH4 from GTP (Werner-Felmayer et al. 2002). To rule out this mechanism, sepiapterin which is converted to BH4 by the pterin salvage pathway independent of the GTP-CHI pathway (Gross and Levi 1992), was added into the culture medium. Sepiapterin did not reverse the inhibitory effect of TGFβ (data not shown), suggesting that the effect of TGFβ is not mediated via BH_4 synthesis.

TGFβ signaling through TGFβ receptors is mediated through Smad-dependent and Smad-independent pathways (Derynck and Zhang 2003, Shi and Massague 2003). Recently, Scharstuhl et al. (Scharstuhl et al. 2003) reported a regulatory role for inhibitory Smad-7 in the biological responses evoked by TGFβ -treatment in the murine H1 chondrocyte cell line. TGFβ activates also the MAPK pathways which mediate some of the cellular responses to TGFβ (Derynck and Zhang 2003). Therefore we studied the role of the MAP kinases in TGFβ -

induced effects on NO production by using pharmacological inhibitors for Erk1/2 (PD98059), p38 (SB203580) and Jnk (SP600126) MAP kinase pathways. None of these inhibitors reversed the inhibitory effects of TGFβ on NO production (data not shown). In addition, TGFβ did not alter IL-1 -induced activation of Jnk, p38 or Erk1/2 kinases (data not shown). The results suggest that the MAPK pathways do not mediate the inhibitory effects of TGFβ on IL-β -induced NO production in H4 chondrocytes.

In the OA process, TGFβ is regarded mainly as a repair factor since it enhances the production of extracellular matrix proteins and stimulates chondrocyte proliferation, but it may also lead to osteophyte formation and fibrosis (Grimaud et al. 2002). The present results point to another mechanism for the anabolic effects of TGFβ on OA cartilage through reduced iNOS expression and NO production. However, the effects of TGFβ on iNOS expression in human chondrocytes or cartilage during different phases of the pathogenesis of OA remain to be studied.

6 Pharmacological regulation of nitric oxide production by disease-modifying antirheumatic drugs

By definition, DMARDs possess the potential to relieve inflammation, to prevent joint damage and to preserve joint integrity and function (Kwoh et al. 2002). Despite the obvious discrepancies between the pathologies of OA and RA, the two diseases share many common features in their inflammatory background leading to cartilage degradation and thus the use of DMARDs for treatment of OA could have beneficial effects on disease progression. DMARDs have shown efficacy in the treatment of rheumatoid arthritis through various molecular mechanisms. Although NO mediates many of the destructive effects of proinflammatory cytokines IL-1 and TNFα in OA and RA, there are only fragmentary data on the pharmacological regulation of NO production by DMARDs.

Anti-TNFα therapy has been shown to reduce iNOS expression and NOS enzyme activity in peripheral blood mononuclear cells this being in conjunction with the changes in the number of tender joints in RA patients in a double-blind, placebo-controlled clinical trial (Perkins et al. 1998). In the present study, the TNFα-antagonists infliximab and etanercept suppressed TNFα -induced iNOS expression and NO production in OA cartilage at clinically achievable drug concentrations (Wyeth, Schering-Plough) indicating, that they are functionally active and penetrate into human OA cartilage. The TNFα antagonists did not inhibit the NO production

induced by IL-1, IL-17 or LPS. This finding suggests that the effect of these cytokines and LPS is not mediated through increased TNFα production in OA cartilage.

Gold compounds have been used successfully in the treatment of RA since 1920, but their precise mechanism of action is still unknown. Gold drugs have shown broad immunomodulatory activities, regulating macrophage phagocytosis, monocyte migration, T and B cell functions, and they can cause changes to levels of peptides and immunoglobulins (Eisler 2003). In the present study, aurothiomalate, a hydrophilic gold salt, inhibited IL-1 -induced NF-κB activation in H4 chondrocytes. This inhibitory effect was reflected in reduced iNOS mRNA and iNOS protein expression, and NO production. Aurothiomalate suppressed IL-1 -induced NO production also in human OA cartilage at clinically achievable drug concentrations (Gerber et al. 1972). Previously, gold compounds have been reported to inhibit NO production in macrophages (Yamashita et al. 1997, Inoue et al. 2001), and the activation of NF-κB in fibroblasts, human lymphocytes and umbilical vein endothelial cells (HUVECs) (Handel 1997, Bratt et al. 2000, Jeon et al. 2000).

Hydroxychloroquine and chloroquine possess anti-inflammatory effects in the treatment of RA, but their mechanisms of action are not fully understood. They seem to inhibit TNFα and IL-1 production, phospholipase A (PLA) and DNA polymerase activity, and to have the capacity to buffer the acidic pH in lysosomes and thus modify antigen processing and presentation by macrophages (Cronstein 1999). In our study, hydroxychloroquine was almost as potent as the selective iNOS inhibitor 1400W at inhibiting IL-1 -induced NO production by human OA cartilage. This was probably due to the suppression of NF-κB activation as shown by EMSA in H4 chondrocytes. The finding is supported by a previous study with murine macrophages, where chloroquine was shown to inhibit IFNγ and LPS -induced NO production and iNOS expression (Hrabak et al. 1998). In the present study, the inhibitory effect was achieved at 100 μM drug concentration, but the safe and clinically achievable blood concentrations remain below 10 μM after oral administration of hydroxychloroquine (Tett et al. 1989). Therefore it is unlikely that systemic use of hydroxychloroquine could inhibit NO production in cartilage. However, intra-articular administration could serve as a possible administration route to obtain sufficient local drug concentrations to inhibit NO production in the cartilage.

Methotrexate is a folic acid antagonist and it inhibits dihydrofolate reductase, interrupting the synthesis of DNA, RNA and proteins, but at the doses used in the treatment of RA the principal mechanism of action seems to be related to inhibition of aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase and thymidylate synthase, and enhanced adenosine release (Cronstein 1999). In relation to NO synthesis, methotrexate inhibits the pterin salvage

pathway, which could interfere with production of tetrahydrobiopterin (BH_4), a cofactor of iNOS (Alderton et al. 2001). In the present study, methotrexate exhibited inhibition of IL-1 -induced NO production, iNOS protein and mRNA expression, and activation of NF-κB in H4 chondrocytes. These results suggest some mechanism other than suppression of BH_4 synthesis. However, methotrexate did not reduce IL-1 -induced NO production in human OA cartilage. The explanation for the differences in the response to methotrexate between murine chondrocytes and human OA cartilage is not clear, but it may result from poor penetration of methotrexate into the cartilage tissue. In addition, inter-species differences in the regulation of iNOS expression have been reported (Kleinert et al. 2003), and may also explain the observed differences in the response to methotrexate treatment between H4 chondrocytes and OA cartilage. Previous studies have shown that methotrexate can inhibit NO production in murine lung epithelial cells, macrophages from rats with adjuvant-induced arthritis, and in bovine chondrocytes (Murrell et al. 1996, Omata et al. 1997, Robbins et al. 1998), whereas it had no effect on NO production in human colon epithelial cells, murine macrophages, rabbit articular chondrocytes or human articular cartilage (De Gendt et al. 1998, Hayem et al. 2000, Hämäläinen et al. 2002). The mechanism by which methotrexate inhibited NO production in those studies remained mostly unknown but it has been reported to suppress NF-κB activation in human Jurkat T-cells, epithelial HeLa cells and myeloid (U937) cells (Majumdar and Aggarwal 2001).

Leflunomide is an immunomodulatory prodrug that is metabolized to active compound A771726 *in vivo*. The active metabolite suppresses lymphocyte proliferation by inhibiting the enzyme dihydroorotate dehydrogenase (DHODH), which leads to interruption of DNA and RNA synthesis due to pyrimidine depletion and subsequent inhibition of inflammatory cell proliferation. In addition, A771726 has been shown to suppress activation of NF-κB (Manna and Aggarwal 1999) and to inhibit tyrosine kinase activity (Chong et al. 1996). In the present experiments, leflunomide inhibited NF-κB activation in H4 chondrocytes, and that was reflected in reduced iNOS mRNA and iNOS protein expression and suppressed NO production. Leflunomide had no effect on NO synthesis in human OA cartilage, but higher concentrations may reduce NO production also in human cartilage (Panico et al. 2003). In activated murine macrophages, both leflunomide and A771726 inhibited NO production, but the IC_{50} values were tenfold higher than the concentration achieved at steadystate plasma concentrations of antirheumatic doses of A771726 (Hamilton et al. 1999).

Taken together, aurothiomalate, hydroxychloroquine, etanercept and infliximab suppressed NO production in OA cartilage indicating that these drugs possess therapeutic potential in the treatment of OA. The inhibitory effect also serves as a mechanism of action of aurothiomalate and TNFα-antagonists to retard progression of RA (Strand and Sharp 2003), because the

inhibitory effects were achieved at drug concentrations found *in vivo* after administration of antirheumatic doses of the drugs.

7 Nitric oxide as a target of drug treatment in osteoarthritis

Osteoarthritis results from a failure to repair the degenerative changes in articular cartilage. The initiator of cartilage catabolism has not been identified, but a number of factors including mechanical stress, cytokines, reactive oxygen species and extracellular matrix components have been suggested. In the OA process, the entire joint structure, the synovial membrane, subchondral bone, ligaments and periarticular muscles, are affected in addition to cartilage. Within the OA joint, the production of proinflammatory and destructive mediators of inflammation, IL-1 and TNFα, along with anabolic factors like TGFβ is enhanced and imbalanced (Pelletier et al. 2001, Goldring and Berenbaum 2004). These mediators act through their receptors and specific signaling pathways, transmit their effect to nucleus, and modulate the transcription of target genes. Pathways leading to cartilage destruction serve as potential targets for developing novel therapies for osteoarthritis (Berenbaum 2004, Malemud 2004, Pelletier 2004).

In OA cartilage, proinflammatory cytokines IL-1 and TNFα enhance the production of NO and it mediates many of the destructive effects of these cytokines in cartilage, e.g. activation of matrix metalloproteinases (MMPs) (Murrell et al. 1995), inhibition of collagen (Cao et al. 1997) and proteoglycan synthesis (Häuselmann et al. 1994, Taskiran et al. 1994, Järvinen et al. 1995), increased susceptibility to injury by other oxidants (e.g. H_2O_2) (Clancy et al. 1997) and chondrocyte apoptosis (Blanco et al. 1995, Hashimoto et al. 1998). NO has also been shown to shift the cytokine balance to a proinflammatory and destructive direction by reducing the synthesis of anabolic TGFβ (Studer et al. 1999) and endogenous IL-1 receptor antagonist (IL-1Ra) by chondrocytes (Pelletier et al. 1996), by contributing to the resistance against anabolic effects of IGF-I (Studer et al. 2000, Loeser et al. 2002) and by increasing the production of the catabolic mediator TNFα by synovial cells (McInnes et al. 1996). These *in vitro* findings suggest, that NO could serve as a pathophysiological target for OA therapy, not only to reduce signs and symptoms of the disease but also to retard disease progression. This hypothesis has been supported by an experimental OA study, where the iNOS inhibitor L-NIL was shown to reduce cartilage erosions, chondrocyte apoptosis, and excess synthesis of IL-1 and MMPs by the synovial membrane and cartilage in a dog model of OA induced with transsection of the anterior cruciate ligament of the knee (Pelletier et al. 1998, Pelletier et al. 1999, Pelletier et al. 2000,

Boileau et al. 2002). However, data from experimental OA models in iNOS-KO mice have produced controversial results. iNOS deficiency has been reported to prevent the degree of cartilage destruction and osteophyte formation in an OA model induced with intra-articular collagenase injection into the knee joint (van den Berg et al. 1999). The opposite result has been reported in an OA model induced with a transsection of the medial collateral ligament and partial medial meniscectomy, which resulted in accelerated development of OA lesions in the cartilage of iNOS KO mice (Clements et al. 2003).

The molecular network in the pathogenesis of OA involves multiple mediators and inhibition of NO production or iNOS deficiency may result in unfavorable compensation mechanisms, which need to be studied. However, current knowledge points to therapeutic potential to iNOS inhibitors as disease-modifying drugs in the treatment of OA and in fact, some of the currently available drugs used in the treatment of arthritis may exert at least part of their therapeutic effects by suppressing NO production. In the present study we investigated mechanisms that regulate iNOS expression and NO production in chondrocyte cultures and in OA cartilage. The results are summarized in Figure 17 and the findings can be applied into the targeted drug development for OA and other inflammatory joint diseases.

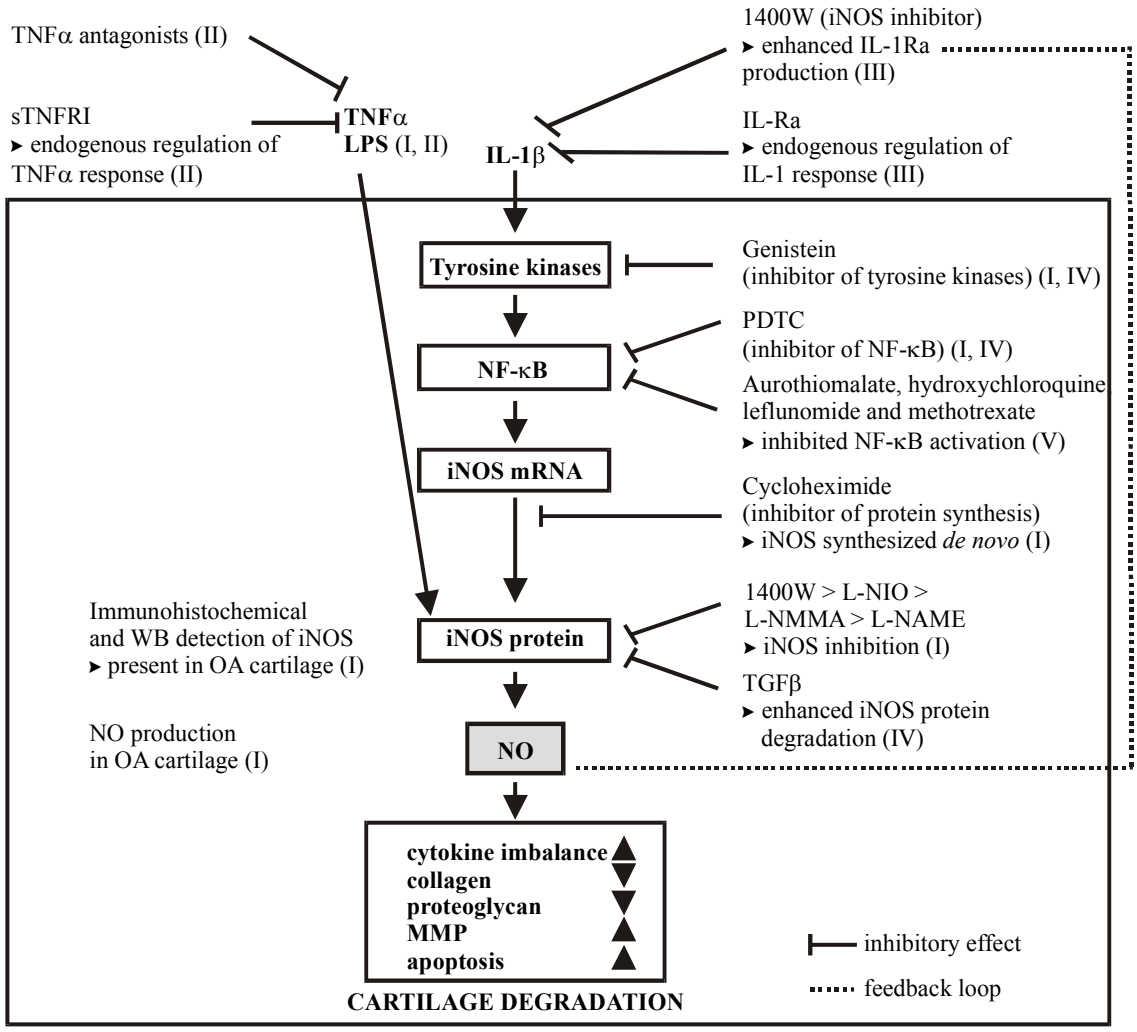


Figure 17. *Summary of the main results on the regulation of iNOS expression in OA cartilage and in H4 chondrocyte culture in the present study. OA cartilage produced NO through the iNOS pathway. IL-1 induced iNOS expression through activation of tyrosine kinases and NF-κB. In addition to IL-1, TNFα and LPS were able to induce the expression of iNOS and NO production. The effects of cytokines were regulated by endogenously released cytokine antagonists sTNFRI and IL-1Ra. The selective iNOS inhibitor 1400W was the most potent inhibitor of NO synthesis and it also reduced the biological activity of IL-1 by stimulating the production of IL-1Ra. TGFβ suppressed NO production in H4 chondrocytes by enhancing iNOS protein degradation. Aurothiomalate, hydroxychloroquine, methotrexate and leflunomide all inhibited IL-1β -induced iNOS expression and NO production in immortalized H4 chondrocytes, probably by suppressing the activation of NF-κB. Aurothiomalate and hydroxychloroquine inhibited IL-1β -induced NO production also in OA cartilage whereas methotrexate and leflunomide had no effect.*

Summary and conclusions

The present study was designed to investigate the regulation of iNOS expression and NO production in chondrocytes in relation to osteoarthritis. Human osteoarthritic cartilage and immortalized H4 chondrocytes were used in the experiments.

The major findings and conclusions were:

1. OA cartilage produced spontaneously NO and this was inhibitable with a selective iNOS inhibitor L-NIO. iNOS expression was detected in OA cartilage. This suggests that OA cartilage produces NO through iNOS.

2. IL-1 is a proinflammatory cytokine found in OA joints. IL-1 induced iNOS expression and NO production in OA cartilage and in H4 chondrocytes. The IL-1 -induced NO production in OA cartilage and in H4 chondrocytes was dependent on activation of tyrosine kinases and NF-κB. Dexamethasone had no effect on IL-1 -induced NO production in OA cartilage and H4 chondrocytes, while it suppressed LPS-induced NO production in J774 macrophages, which were used as controls.

3. OA cartilage is able to regulate the biological activity of IL-1 and TNFα by producing endogenous cytokine antagonists IL-1Ra, sIL-1RII, sTNFRI and sTNFRII. RA cartilage produced IL-1Ra and sIL-1R II in higher concentrations than OA cartilage, which may explain why OA cartilage responded to IL-1-stimulation with higher NO production compared to RA cartilage. The studies with neutralizing antibodies against sTNFRs suggest that endogenous cartilage-derived TNFα-antagonists (sTNFRI) modulate NO production in OA cartilage.

4. IL-1 stimulated IL-1Ra production in OA cartilage and this was further enhanced by a highly selective iNOS inhibitor 1400W. This result suggests that NO can down-regulate IL-1Ra production and thus enhances the destructive effects of IL-1 in cartilage.

5. TGFβ inhibited IL-1 -induced NO production and iNOS protein expression, but failed to have any effect on iNOS mRNA in H4 chondrocytes. Degradation of iNOS protein was enhanced in cells treated with a combination of IL-1 and TGFβ as compared to IL-1 -treated cells. This finding points to an additional mechanism for TGFβ to counteract the destructive effects of IL-1 in OA and the importance of post-transcriptional mechanisms in the regulation of NO production in chondrocytes.

6. The TNFα-antagonists, etanercept and infliximab, suppressed TNFα-induced NO production in OA cartilage but had no effect on NO production induced by IL-1, LPS or IL-17. This data suggests that TNFα is not an autacoid mediator in these processes. Since TNFα has been found in inflamed joints, some of the anti-inflammatory and antierosive mechanisms of TNFα antagonists may well be related to their inhibitory effect on NO production in the cartilage.

7. Aurothiomalate and hydroxychloroquine suppressed IL-1 -induced NO production in chondrocyte cultures and in OA cartilage offering therapeutic potential for these drugs in the treatment of OA. The inhibitory effect also serves as a mechanism of action of aurothiomalate to retard progression of RA, because the inhibitory effect was achieved at drug concentrations found *in vivo* after administration of antirheumatic doses.

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