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Cyclo-oxygenase-2, MAP Kinase Pathways and Aurothiomalate

Induction of MAP kinase phosphatase-1
as a novel mechanism of action of anti-rheumatic
drug aurothiomalate

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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-IV.

I   Nieminen R, Leinonen S, Lahti A, Vuolteenaho K, Jalonen U, Kankaanranta H, Goldring MB and Moilanen E (2005): Inhibitors of mitogen-activated protein kinases down-regulate COX-2 expression in human chondrocytes. Mediat Inflamm 2005:249-255.

II   Nieminen R, Lahti A, Jalonen U, Kankaanranta H and Moilanen E (2006): JNK inhibitor SP600125 reduces COX-2 expression by attenuating mRNA in activated murine J774 macrophages. Int Immunopharmacol 6:987-996.

III   Nieminen R, Vuolteenaho K, Riutta A, Kankaanranta H, van der Kraan PM, Moilanen T and Moilanen E (2008): Aurothiomalate inhibits COX-2 expression in chondrocytes and in human cartilage. Effects on COX-2 mRNA stability. Eur J Pharmacol 587:309-316.

IV   Nieminen R, Korhonen R, Clark AR, Moilanen T and Moilanen E: DMARD aurothiomalate inhibits COX-2, MMP-3 and IL-6 expression in chondrocytes by increasing MKP-1 expression and decreasing p38 phosphorylation. MKP-1 as a novel target for antirheumatic drugs. Submitted for publication.

Abbreviations

AP	activator protein
ARE	AU-rich element
AUF	ARE/poly(U)-binding/degradation factor
cAMP	3'-5'-cyclic adenosine monophosphate
C/EBP	CCAAT-enhancer box binding protein
CRE	cAMP response element
COX-2	cyclooxygenase-2
cPLA	cytosolic phospholipase
DMARD	disease-modifying antirheumatic drug
DUSP	dual specificity phosphatase
ELISA	enzyme linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinase 1/2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HuR	Hu antigen R
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
KO	knockout, gene deletion
LO	lipoxygenase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MK	MAPK-activated protein kinase, MAPKAPK
MKK	MAPK kinase
MKK1/2	MAPK/ERK kinase 1 and 2, MEK1/2
MKKK	MAPK kinase kinase
MKP	MAPK phosphatase
MMP	matrix metalloproteinase
NF-κB	nuclear factor-κB
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
PD98059	2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one, MKK1/2 inhibitor, inhibits activation of ERK1/2 kinase
PG	prostaglandin
PGES	prostaglandin E synthase
RA	rheumatoid arthritis
RT-PCR	reverse transcriptase/real time PCR

SB202474   4-ethyl-2-(4-methoxyphenyl)-5-(4-hydroxyphenyl)-5-(4pyridyl)-imidazole, negative control compound for SB203580
SB203580   4-(4-flurophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazol, p38 inhibitor
siRNA   small interfering RNA
SP600125   anthra(1,9-cd)pyrazol-6(2H)-one, JNK inhibitor
TGF   transforming growth factor
TLR   toll-like receptor
TNF-α tumor necrosis factor α
TTP   tristetraprolin
Tx   thromboxane
3'UTR   3'-untranslated region of mRNA

Abstract

Prostaglandins (PGs) are ubiquitously produced and regulate various physiological and pathophysiological responses in the human body. In inflammatory joint diseases including rheumatoid arthritis (RA) and osteoarthritis (OA) prostaglandins have regulatory and proinflammatory effects and mediate inflammatory pain. Prostaglandins are formed from arachidonic acid in a reaction catalyzed by cyclooxygenase (COX) enzymes. Two forms of COX enzyme have been identified: constitutive isoform COX-1 and inducible isoform COX-2. COX-2 is induced in response to proinflammatory cytokines and bacterial products like endotoxins and is mainly responsible for the production of inflammatory prostaglandins (especially PGE_2). In inflammatory arthritis, COX-2 expression is highly enhanced in chondrocytes and in the synovial tissue. In the joints, prostanoids produced by COX-2 pathway can exert catabolic or anabolic effects in the cartilage depending on the microenvironment, and modulate inflammation and inflammatory pain.

The aim of the present study was to investigate the role of mitogen-activated protein kinases (MAPKs) in the regulation of COX-2 expression and PGE_2 production in chondrocytes and in intact cartilage as potential targets for drug development. Another major aim was to investigate the effects of disease modifying anti-rheumatic drugs (DMARDs) on COX-2 expression and further to study the mechanisms of action of aurothiomalate.

Inhibitors of p38 and JNK MAPK pathways were found to regulate COX-2 expression and PGE_2 production by facilitating COX-2 mRNA degradation. Interestingly, also a DMARD aurothiomalate inhibited COX-2 expression and PGE_2 production in chondrocytes and in human cartilage by destabilizing COX-2 mRNA. In addition, aurothiomalate also reduced levels of

phosphorylated (i.e. active) p38 MAPK and increased MAPK phosphatase (MKP)-1 expression. MKP-1 is an endogenous regulator of MAPK pathways, and it is known to inactivate p38 MAPK by dephosphorylation. In addition to COX-2 expression and PGE_2 production, aurothiomalate inhibited also the production of inflammatory cytokine interleukin (IL)-6 and matrix metalloproteinase (MMP)-3 which is an enzyme involved in the degradation of cartilage matrix. Silencing of MKP-1 by short interfering RNA significantly impaired the ability of aurothiomalate to inhibit the phosphorylation of p38 MAPK and the expression of COX-2, MMP-3 and IL-6. Similarly, aurothiomalate reduced COX-2, MMP-3 and IL-6 expression in human RA cartilage and in articular cartilage from wild type mice but not in cartilage from MKP-1 deficient mice.

The findings provide a novel mechanism of action for aurothiomalate through increased MKP-1 expression, reduced p38 MAPK activation and suppressed expression of COX-2, MMP-3 and IL-6 that may at least in part explain the anti-inflammatory and anti-erosive action of aurothiomalate. MKP-1 may therefore be a promising novel target for the development of disease modifying drugs for inflammatory joint diseases.

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Tiivistelmä

Prostaglandiinit säätelevät monia elimistön fysiologisia ja patofysiologisia toimintoja. Useissa tulehduksellisissa nivelsairauksissa, kuten nivelreumassa ja nivelrikossa, prostaglandiineilla tiedetään olevan tulehdusta ja kipua voimistavia vaikutuksia. Prostaglandiinit muodostuvat arakidonihaposta syklo-oksigenaasientsyymin (COX) katalysoimassa reaktiossa. COX entsyymistä tunnetaan kaksi muotoa: konstitutiivinen COX-1 ja indusoituva COX-2. Bakteeriperäiset tuotteet, esimerkiksi endotoksiinit, ja tulehdusta voimistavat sytokiinit lisäävät COX-2:n ilmentymistä sekä tulehdusta ja kipua voimistavien prostaglandiinien, kuten prostaglandiini (PG) E_2:n tuottoa. Tulehtuneessa nivelessä COX-2:n tiedetään ilmentyvän voimakkaasti kondrosyyteissä ja synoviakudoksessa. COX:n katalysoimissa reaktioissa muodostuneet prostanoidit voivat mikroympäristöstä riippuen säädellä ruston katabolisia tai anabolisia toimintoja sekä voimistaa tulehdusta ja kipua.

Väitöskirjatyön tarkoituksena oli tutkia mitogeenien aktivoimien proteiinikinaasien (MAPK) roolia COX-2 entsyymin ilmentymisen ja tätä seuraavan PGE_2 tuoton säätelyssä kondrosyyteissä ja rustossa. Lisäksi tutkittiin antireumaattisten lääkkeiden vaikutusta COX-2 entsyymin ilmentymiseen ja PGE_2 tuottoon. Tutkituista antireumaattisista lääkkeistä tehokkaimmaksi COX-2 entsyymin ilmentymisen ja PGE_2 tuoton estäjäksi osoittautui aurotiomalaatti, jonka molekulaarista vaikutusmekanismia tutkittiin tarkemmin.

Tulehduksessa tärkeimmät MAPK reitit ovat p38 ja JNK. Näiden estäjien havaittiin säätelevän COX-2 entsyymin ilmentymistä ja PGE_2:n tuottoa nopeuttamalla COX-2:n lähetti-RNA:n hajoamista. Myös antireumaattisen lääkeaineen, aurotiomalaatin, havaittiin estävän COX-2 entsyymin ilmentymistä ja PGE_2:n tuottoa nopeuttamalla COX-2:n lähetti-RNA:n hajoamista

kondrosyyteissä ja rustossa. Tämän lisäksi aurotiomalaatin havaittiin vähentävän p38 kinaasin fosforylaatiota ja lisäävän p38 kinaasia inaktivoivan MAPK fosfataasi (MKP)-1:n tuottoa soluissa. COX-2:n eston lisäksi aurotiomalaatin havaittiin estävän rustotuhoa välittävän matriksin metalloproteaasi (MMP)-3:n sekä tulehduksellisen sytokiinin IL-6:n tuottoa kondrosyyteissä ja rustossa. Käyttäen siRNA tekniikkaa ja MKP-1 poistogeenisten hiirten rustoa, todettiin, että aurotiomalaatti esti COX-2 välitteistä prostaglandiinituottoa sekä tulehdustekijöiden MMP-3 ja IL-6 tuottoa lisäämällä MKP-1 fosfataasin synteesiä.

Tutkimuksessa löydettiin pitkään käytössä olleelle tehokkaalle reumalääkkeelle, aurotiomalaatille, täysin uusi molekulaarinen vaikutusmekanismi, joka voi selittää sen tulehdusta ja niveltuhoa lievittäviä vaikutuksia. Tietoa voidaan käyttää hyväksi kehitettäessä uusia tehokkaita lääkkeitä tulehduksellisten nivelsairauksien hoitoon.

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Introduction

Inflammation is body's protective attempt to remove injurious stimuli as well as to start the healing process of damaged tissue. It is a complex, tightly regulated response against harmful stimuli e.g. pathogens, damaged cells or irritants. If the regulation fails or if the reaction turns against its own tissues, inflammation can lead to chronic inflammatory diseases like rheumatoid arthritis (RA). Inflammation can be classified being either acute or chronic. Acute inflammation is the initial response to harmful stimuli and it is characterized by the increased movement of plasma and leukocytes from the blood into the injured tissues. A cascade of events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. The ideal outcome of acute inflammation is the reversion of injured tissue back to normal. Chronic inflammation is characterized by the presence of inflammatory cells, mainly macrophages, lymphocytes and plasma cells within the injured tissue. In its chronic state, the inflammation is persistent and there is simultaneous tissue destruction and attempted repair.

Prostaglandins (PG) are present in a wide variety of human tissues, where they play a central role in inflammation and regulate many physiological responses, including vascular tone, blood clotting, kidney function, gastric secretion and reproduction. Cyclooxygenase-2 (COX-2) is the key enzyme in the synthesis of PGs in inflammation. (Turini and Dubois 2002) COX-2 is highly expressed in chondrocytes and in the synovial tissue of patients with arthritis (Siegle et al. 1998). Prostanoids produced by the COX-2 pathway mediate inflammation and inflammatory pain, and they can exert catabolic or anabolic effects in the cartilage depending on the microenvironment (Amin et al. 1999, Goldring and Berenbaum 2004). Prostanoids and COX-inhibitors have been reported to regulate collagen production, proteoglycan turnover, and the production of

matrix metalloproteinases in cartilage (Hardy et al., 2002, Fernandez et al., 2004, Goldring and Berenbaum 2004, Mastbergen et al. 2006). In addition, increased COX-2 expression seems to mediate shear stress –induced chondrocyte apoptosis (Healy et al. 2005).

Mitogen-activated protein kinases (MAPKs) are a family of protein kinases, which are part of the signaling cascade mediating the transmission of inflammatory signals to intracellular target proteins. MAPKs are known to regulate many cellular responses e.g. cytokine production, cell proliferation and apoptosis. MAPKs have to be phosphorylated before they can exert kinase activity; MAPK phosphatases (MKPs) are endogenous regulatory pathways which inactivate MAPKs via dephosphorylation. (Dong et al. 2002)

RA is a chronic, progressive disease that is characterized by inflammation in the synovial membrane. It principally affects joints causing substantial damage and disability due to inflammation and pain, as well as the destruction of cartilage, bone and other joint structures. Generalized inflammation in other tissues such as lungs, heart, muscles and skin may also complicate the disease. RA is associated with severe morbidity, functional decline, permanent disability, and an increase in mortality. The etiology of RA is unclear, but inflammation is a key pathogenetic mechanism involved in joint destruction and determining symptoms and disability. The treatment of RA is based on early, aggressive therapy with disease-modifying antirheumatic drugs (DMARDs) to decrease the disease activity and prevent joint erosion. Non-steroidal anti-inflammatory drugs (NSAIDs) and anti-inflammatory steroids are used to suppress the symptoms. (Feldmann et al. 1996a)

The results of the present study offer a novel mechanism for the anti-inflammatory and anti-erosive action of a DMARD, aurothiomalate, and provide new targets for the drug development of arthritis.

Review of the literature

1. Eicosanoids

The first publications about fatty acid derivatives, later known as eicosanoids (from the Greek *eicosa* meaning twenty; for derivatives of twenty carbon fatty acids) were published in 1930. Firstly, Burr and Burr reported that when rats were fed without fat, this evoked growth deceleration, reproductive problems, scurfy skin, damage in kidney, and inordinate drinking (Burr and Burr 1930). Secondly, Kurzrok and co-workers depicted a factor that has fatty acid properties and was a vasodepressor and a smooth muscle-stimulant (Kurzrok and Lieb 1930). This factor was named prostaglandin after the prostate gland where it was thought to be synthesized (von Euler 1935, Goldblatt 1935). The first prostaglandin subfamilies were marked with letters E and F because of their partition between ether and phosphate buffer (fosfat in Swedish); the E-types tended to remain extracted in ether whereas the F-types could be extracted in phosphate buffer (Bergström and Sjövall 1957). In 1964, Bergström and Samuelsson showed that prostaglandins were produced from a fatty acid, arachidonic acid (Bergström et al. 1964). The eicosanoid story continued in 1975 when Samuelsson and co-workers found the platelet aggregator, thromboxane A_2 (TxA_2) (Hamberg et al. 1975). One year later Moncada, Vane and co-workers described prostacyclin (PGI_2) that was a potent inhibitor of platelet secretion and aggregation (Moncada et al. 1976). The leukotrienes were discovered in 1979 by Samuelsson and co-workers (Samuelsson et al. 1979).

Eicosanoids are a group of lipid mediators formed after oxidation of the 20-carbon fatty acid, arachidonic acid. They are critical regulators of several physiological and pathophysiological responses and coordinate cellular events towards proper tissue function. Eicosanoids are also recognized for their role in

inflammation, fever and pain. (Murphy et al. 2004) The major mechanism of action of non-steroidal anti-inflammatory drugs (NSAIDs) is the inhibition of prostaglandin H synthase (PGHS, usually known as COX that is a short form of cyclooxygenase) resulting in a reduced synthesis of eicosanoids (Vane 1971, Warner and Mitchell 2004). Glucocorticoids also reduce eicosanoid synthesis but their mechanism of action is more complex. Glucocorticoids have been reported to induce the synthesis of lipocortins that are inhibitors of cytosolic phospholipase $(cPLA)_2$ which releases arachidonic acid from cell membrane phospholipids (Di Rosa et al. 1984). Glucocorticoids are also able to down-regulate expression of COX-2 and microsomal PGE synthase (mPGES)-1 (Raz et al. 1989, Ristimäki et al. 1996, Thorén and Jacobsson 2000).

1.1 Biosynthesis of eicosanoids

Arachidonic acid, which is released from cell membrane phospholipids by cPLA, is the premier eicosanoid precursor in mammalian cells. Subsequently, arachidonate is metabolized either by the 5-lipoxygenase (5-LO) pathway into leukotrienes (LTs) or by the COX pathway into PGs. Eicosanoids function as autocrine and paracrine lipid mediators by targeting specific 7-transmembrane G-protein coupled receptors. The signal is mediated via changes in intracellular cAMP, calcium, or diacylglyerol, depending on the type of associated G-protein. (Murphy et al. 2004)

Prostanoids, including PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TxA_2 are synthesized by most cells in the human body. The cells do not store prostaglandins; they are synthesized *de novo* from membrane-released arachidonic acid in response to cellular activation, such as mechanical trauma, cytokines, growth factors or a number of other (often cell specific) stimuli (e.g. bradykinin and thrombin in endothelium). (Murphy et al. 2004)

Although several enzymes have been shown to regulate the cellular levels of free arachidonic acid, $cPLA_2$ seems to be the most important in this respect. Cells lacking $cPLA_2$ do not normally display eicosanoid synthesis. The activity of

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$cPLA_2$ is regulated by translocation from cytosol to membrane phospholipids which in turn is regulated by cell-specific and agonist-dependent events. (Evans et al. 2001) At the endoplasmic reticulum and nuclear membrane, arachidonic acid that is released by $cPLA_2$ is presented to COX enzymes which transform it to PG endoperoxides PGG_2 and PGH_2 which, in turn, are metabolized to prostanoids by specific synthases, i.e. Tx synthase, PGI synthase, PGF synthase, PGD synthase and PGE synthase.

Two isoforms of COX are known: COX-1 and COX-2. COX-1 can be regarded as the physiological enzyme that is responsible for basal, constitutive prostaglandin synthesis, whereas COX-2 is important in various inflammatory and other pathological conditions (Table 1).

Table 1. *Comparison of cyclooxygenase enzymes.*

	COX-1	COX-2
Gene locus	9q32-q33.3	1q25.2-q25.3
Size of gene	22 kb	8.3 kb
Molecular weight	70 kDa	72 kDa
Mode of expression	Constitutive	Inducible

In the reactions catalyzed by COX enzymes, arachidonic acid is first oxygenated to an endoperoxide-containing PGG_2 that is later converted to PGH_2 by a reduction of the hydroperoxyl moiety. COX-1 and COX-2 are very similar in their crystal structures. There are also differences, e.g. the catalytic site of COX-2 is located in a wider channel than that of COX-1; bulky COX-2 inhibitors do not fit into the COX-1 channel. In the catalytical site, the main difference between COX-1 and COX-2 is at amino acid 523, where the former contains isoleucine, the latter valine. The presence of valine allows methylation, which leads to a type of "side pocket" formation in COX-2. This methylated side pocket has been targeted during development of COX-2 inhibitors (Hawkey et al. 1999). The structure and main differences in the active sites of COX-1 and COX-2 enzymes are presented in Figure 1.
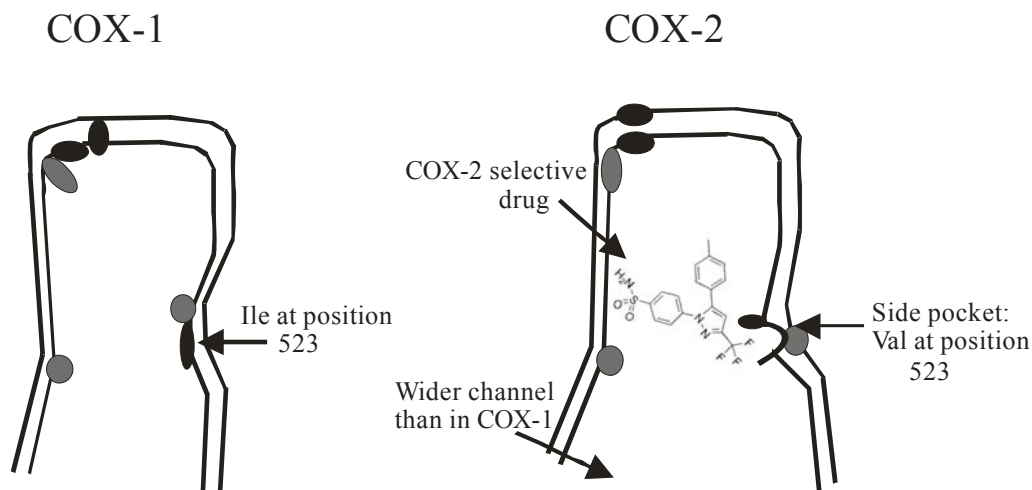
Figure1. *A schematic figure of the catalytic site of COX-1 and COX-2 enzymes. Ile=isoleucine, Val= valine. (Modified from Hawkey et al. 1999.)*

Downstream of PGH_2 are the specific prostanoid synthase enzymes which are expressed in a cell specific manner. Tx synthase is found in platelets and macrophages, PGI_2 synthase is located in endothelial cells and PGF synthase in uterus. In addition to these enzymes, two types of PGD synthases are found, one in brain and one in mast cells. (Coleman et al. 1994). Prostaglandin E synthase (PGES) catalyzes the conversion of the COX-product PGH_2 to PGE_2. In 1999 it was shown that this enzyme belonging to membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) –family was responsible for PGE_2 biosynthesis. (Jakobsson et al. 1999) Three distinct PGES isoforms have been identified. Cytosolic PGES (cPGES) is ubiquitously and constitutively expressed, and displays functional coupling with COX-1. In contrast, the microsomal PGES-1 (mPGES-1) is an inducible enzyme that exhibits functional coupling with COX-2. The most recently identified isoform, mPGES-2, is ubiquitously expressed in diverse tissues, but its function and regulation remain obscure. (Fahmi 2004, Samuelsson 2007)

Many reports about high-affinity binding sites for prostanoids in various cells and tissues were published in the early 1970s (Kuehl and Humes 1972, Powell et al. 1974, Rao 1974a, Rao 1974b). Kennedy and co-workers integrated the information from earlier studies and published a novel classification of prostanoid receptors in 1982. They proposed that the name of each receptor

contains letter P which is preceded by a letter of the most potent natural prostanoid agonist at that receptor. The nomenclature of receptors for PGD, PGE, PGF, PGI and Tx become DP-, EP-, FP-, IP- and TP-receptors, respectively. (Kennedy et al. 1982) EP receptors have been further divided into four subtypes EP_1, EP_2, EP_3 and EP_4, based first on the differences in cellular responses to PGE_2 and the use of selective agonists and antagonists (Coleman et al. 1994) and subsequently all four subtypes of EPs have been identified and cloned (Sugimoto and Narumiya 2007). (Figure 2)
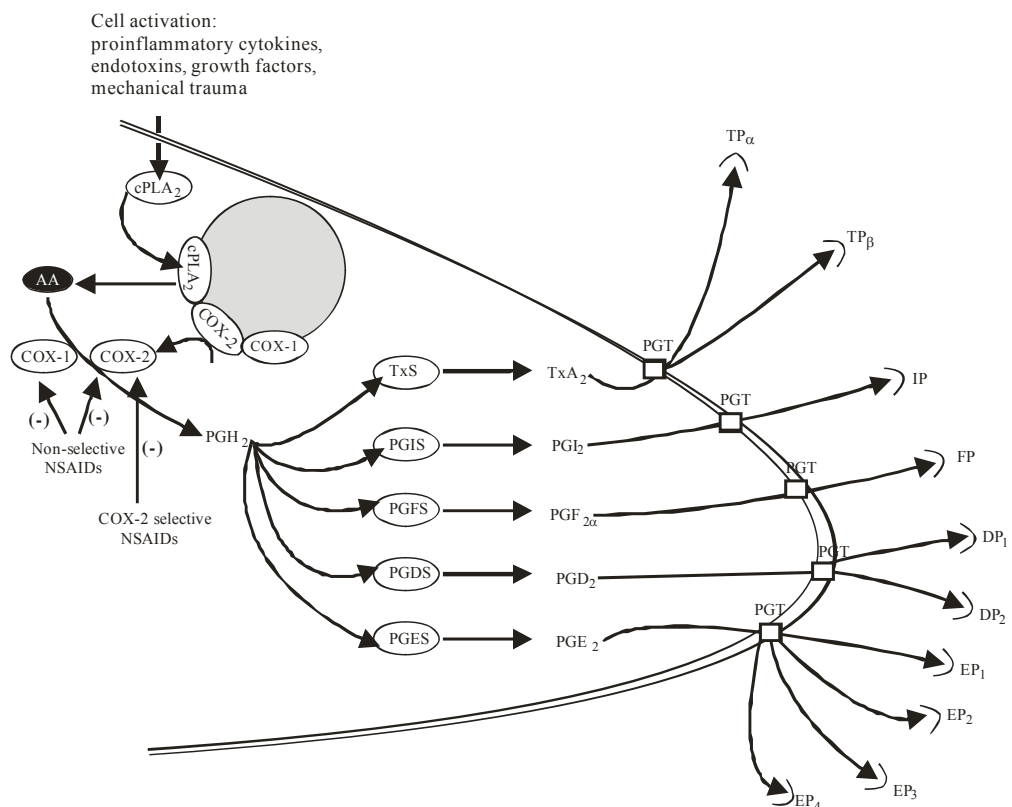
Figure 2. *A schematic figure of prostaglandin synthesis and prostaglandin receptors. When cells are activated (e.g. by mechanical trauma, cytokines, growth factors or various inflammatory stimuli), cytosolic phospholipase ($cPLA_2$) translocates to endoplasmic reticulum and nuclear membrane and arachidonic acid (AA) is released from cell membrane phospholipids. PGH_2 is formed in reactions catalyzed by COX-1 and COX-2. Traditional NSAIDs inhibit both COX-1 and COX-2 whereas COX-2 selective NSAIDs inhibit only COX-2 enzyme.*
TxS=thromboxane synthase, PGIS=PGI synthase, PGFS=PGF synthase, PGDS=PGD synthase, PGES=PGE synthase, PGT=prostaglandin transporter, TP=thromboxane receptor, IP=PGI receptor, FP=PGF receptor, DP=PGD receptor, EP=PGE receptor

In contrast to prostaglandins, only inflammatory cells such as polymorphonuclear leukocytes, macrophages and mast cells principally produce leukotrienes. $cPLA_2$ and 5-LO translocate to the nuclear envelope in response to cellular activation e.g. by the presence of immune complexes or bacterial products. 5-LO is the most important enzyme in leukotriene synthesis. It is a nonheme iron dioxygenase that is located in the nucleus or in the cytosol of the cell. (Peters-Golden and Brock 2001) 5-LO possesses a NH_2-terminal domain that binds two calcium ions, and a large catalytic domain that binds iron (Chen and Funk 2001). 5-LO acts by transforming the arachidonic acid released to LTA_4. There are three circumstance-dependent possibilities to continue from LTA_4: hydrolysis, glutathione conjugation or transcellular metabolism to generate bioactive eicosanoids. LTB_4, which is a potent neutrophil chemoattractant and a stimulator of leukocyte adhesion to endothelial cells, is formed mainly in the cytoplasm via the hydrolysis by the enzyme leukotriene A_4 hydrolase (LTA_4H). Cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) are known from their slow and sustained smooth muscle contracting abilities. LTC_4 is formed in a reaction catalyzed by LTC_4 synthase (LTC_4S) in which LTA_4 is conjugated with glutathione. LTC_4 is transported out of the cell and its peptide moiety is subjected to extracellular metabolism; this is the route by which LTD_4 and LTE_4 are formed. (Gronert et al. 1999) Leukotriene synthesis is presented in Figure 3.
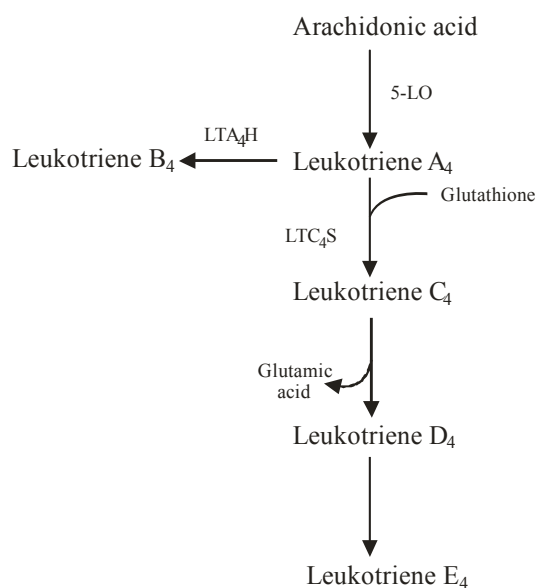
Figure 3. *A Schematic figure of leukotriene synthesis.* *5-LO=5-lipoxygenase, LTA_4H=leukotriene A_4 hydrolase, LTC_4S=leukotriene C_4 synthase.*

1.2 Role of prostaglandins in inflammation

Local redness, swelling, heat and pain are the classical signs of acute inflammation. Reddening, swelling and heat generation occur in response to increased blood flow and vascular permeability. Previously, it has been shown that PGs play a role in the inflammatory symptoms due to vasodilatation synergistically with other mediators (including bradykinin and histamine) to enhance blood flow, increase vascular permeability and evoke edema. PGE_2 and PGI_2 seem to be the most powerful prostaglandins mediating inflammation and they both can be found in high concentrations at sites of inflammation. (Davies et al. 1984, Fleming and Kelly 2004)

COX-2 is highly expressed in chondrocytes and in the synovial tissue of arthritis patients (Siegle et al. 1998). Prostanoids produced by COX-2 pathway mediate inflammation and inflammatory pain, and they can exert catabolic or anabolic effects in the cartilage depending on the microenvironment (Amin et al. 1999, Goldring and Berenbaum 2004). Prostanoids and COX-inhibitors have been reported to regulate collagen production, proteoglycan turnover, and matrix metalloproteinase production in cartilage (Hardy et al. 2002, Fernandez et al. 2004, Goldring and Berenbaum 2004, Mastbergen et al. 2006).

PGE_2 has also a mediator role in fever. EP_3-deficient mice unlike the other EP receptor knockouts did not show febrile responses after challenge with PGE_2, IL-1β or lipopolysaccharide (LPS) (Ushikubi et al. 1998). Interestingly, also COX-2 deficient mice have revealed a decreased febrile response after LPS (Li et al. 1999).

PGE_2 acts as a vasodepressor through the EP_2 and EP_4 receptors and plays a major role in acute inflammation by acting on the peripheral circulation and inducing hyperemia and swelling (Audoly et al. 1999, Zhang et al. 2000). Interestingly, vascular smooth muscle constriction has been reported to be mediated by EP_1 and / or EP_3 receptors (Audoly et al. 2001, Jadhav et al. 2004). This suggests that the balance between the functional activities of distinct EP

receptors may determine the effect of prostaglandin E_2 on the vasculature. The role of PGI_2 in inflammatory swelling was tested in a carrageenan-induced paw swelling –model. IP-deficient mice developed swelling only to similar levels as indomethacin-treated wild-type mice and indomethacin treatment did not decrease the swelling in IP-deficient mice. (Murata et al. 1997)

The studies into the role of prostaglandins in pain have indicated that PGE_2, PGE_1 and PGI_2 exert stronger effects than other PGs. This points to participation of EP or IP receptors in enhancing inflammatory pain. (Bley et al. 1998) EP_3 knockout mice were shown to have a decreased writhing response in an acetic acid –induced writhing model where mice were pretreated with LPS (Ueno et al. 2001). Monoclonal antibody against PGE_2 has been shown to inhibit phenylbenzoquinone –induced writhing in mice and carrageenan –induced paw hyperalgesia in rats similarly as indomethacin (Mnich et al. 1995, Portanova et al. 1996). Unlike EP knockout mice, IP knockout mice did not show any changes in their nociceptive reflexes when these were evaluated by hot plate and tail flick tests. This suggested that IP is not involved in nociceptive neurotransmission at the spinal and supraspinal levels. In the acetic acid –induced writhing test, IP-deficient mice showed significantly less responses than wild-type animals. In addition to these results, in wild-type mice, intraperitoneal injections of PGE_2 or PGI_2 induced responses whereas IP-deficient mice showed responses only to PGE_2. (Murata et al. 1997) In summary, these results indicate that the pain sensation can be modulated by both EP and IP receptors.

The main site of hyperalgesic prostanoid action is located in the periphery where prostaglandins are considered to sensitize the free ends of sensory neurons. EP_1-, EP_3-, EP_4- and IP-receptors have been found in sensory neurons, and PGE_2 and PGI_2 seem to be important mediators of hyperalgesia (Higgs et al. 1983, Oida et al. 1995). The EP_3 receptor has been shown to mediate hyperalgesia; in EP_3 deficient mice intrathecal injection of PGE_2 produced hyperalgesia at higher doses than in wild type mice (Minami et al. 2001). Spinal injection of a COX-inhibitor has been shown to inhibit thermal hyperalgesia that is induced by the activation of spinal glutamate and substance P receptors (Malmberg and

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Yaksh 1992). IL-1β has been shown to be the major inducer of COX-2 upregulation in the central nervous system. Intraspinally administered interleukin-converting enzyme or a COX-2 inhibitor decreased inflammation-induced central PGE_2 production and mechanical hyperalgesia. (Samad et al. 2001). PGs in the spinal cord are also believed to be part of the development of allodynia. In one study, EP_1-deficient mice did not display allodynia after PGE_2 induction whereas wild-type mice and mice lacking EP_3 exhibited allodynia in response to PGE_2 (Minami et al. 2001).

In addition to pro-inflammatory actions, prostanoids have also been reported to exert anti-inflammatory responses. The anti-inflammatory actions of prostanoids have been seen in allergic or immune inflammation and are usually linked with pro-inflammatory actions of other prostanoids. One example of this is the antagonism between PGD_2 –DP and PGE_2 – EP3 pathways in elicitation of allergic asthma (Matsuoka et al. 2000, Kunikata et al. 2005).

2. Regulation of COX-2 expression and PGE_2 production

The cyclooxygenase reaction through which arachidonic acid is enzymatically converted to PGs was identified by Samuelsson and colleagues in the early 70s (Hamberg and Samuelsson 1973, Hamberg et al. 1974). The breakthrough in prostaglandin research occurred in 1971 when it was shown that commonly used NSAIDs inhibit the biosynthesis of prostaglandins (Ferreira et al. 1971, Smith and Willis 1971, Vane 1971). At the beginning of the 1980s, many research laboratories speculated about the existence of more than one COX enzyme. Habenicht and colleagues reported in 1985 about two-peak induction in prostaglandin synthesis (Habenicht et al. 1985). This study was indicative of the existence of constitutive and inducible forms of COX enzyme. In 1990, a study with LPS stimulated macrophages concluded "cells may contain two pools of COX, each with a differential sensitivity to LPS or dexamethasone" (Masferrer et al. 1990). Subsequently, COX-2 was discovered in 1991, as a primary response gene (Kujubu et al. 1991, Xie et al. 1991).

COX-2 expression is regulated both by transcriptional and post-transcriptional mechanisms. The human COX-2 gene is located on chromosome 1 q25.2-q25.3 (Inoue et al. 1995). It is about 8.3 kb long and has 10 exons. Three transcripts of COX-2 have been described: 2.8, 4.0 and 4.6 kb in length. (Hla and Neilson 1992, Jones et al. 1993)

2.1 Transcriptional regulation of COX-2

Sequence analysis of the 5'-flanking region of COX-2 gene reveals that there are many consensus *cis*-elements that regulate the transcription of COX-2. However, in all species studied, only a limited number of elements are known to be involved in the regulation of COX-2 gene expression, i.e. the cAMP response element (CRE), the C/EBP-NF-IL6 (CAAT/enhancer binding protein), NF-κB (nuclear factor κB) and NFAT (nuclear factor of activated T cells) sites and the E-box. (Klein et al. 2007) There are some interspecies differences in the sequences of human and murine COX-2 genes. For example, the murine COX-2 promoter has one NF-kB motif and two C/EBP sites instead of the two NF-kB sites and one C/EBP motif found in human COX-2 promoter. (Tanabe and Tohnai 2002) The mechanisms of transcriptional regulation of COX-2 vary extensively depending on the stimulus and cell type.

The CRE has been identified as one of the most important regulatory elements in the COX-2 promoter region (Tanabe and Tohnai 2002). Homo- or hetero-dimers of c-fos, c-jun, ATF family members on bZIP proteins and the cAMP response element binding protein (CREB) can bind to this CRE response element and activate COX-2 expression (Nakabeppu et al. 1988, Rauscher et al. 1988, Du et al. 1993, van Dam et al. 1993). The importance of CRE has been demonstrated in mutation studies. When both CRE and NF-IL-6 regulatory elements were mutated, the expression of COX-2 was reduced by 75 % in human endothelial cells in response to TPA and LPS treatment, whereas destruction of only the NF-IL-6 motif or CRE caused inhibition of 40 and 10 %, respectively (Inoue et al. 1995). In human endothelial cells and fibroblasts, mutation of the CRE site

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converted COX-2 promoter into being unresponsive to IL-1β, TNF-α, PMA and prostaglandins (Schroer et al. 2002). In the human COX-2 promoter, the CRE and E-box elements overlap (Tanabe and Tohnai 2002). CRE is known to synergize with NF-κB and NF-IL-6 to a greater extent than the E-box to induce COX-2 gene transcription (Mestre et al. 2001).

The expression of COX-2 requires the presence of at least one of the two NF-IL-6 motifs to be present in the promoter (Wadleigh et al. 2000). Activation at the NF-IL-6 motif is often linked with C/EBP transcription factors that can either induce or repress COX-2 gene expression (Kim and Fischer 1998).

There are two NF-κB consensus sites in the human COX-2 promoter. NF-κB is known to be activated by many pro-inflammatory factors and it has been shown to be involved in the regulation of COX-2 in many, but not all, cell types (Crofford 1999, Ke et al. 2007). Interestingly, NF-κB may be inhibited by some NSAIDs, such as acetylsalicylic acid. These drugs are able to target and activate the inhibitory element of NF-κB, i.e. IκB, and thus inhibit NF-κB –mediated signaling. (Kopp and Ghosh 1994, Cavallini et al. 2001) It is noteworthy that the salicylate concentrations needed to inhibit NF-κB are relatively high (mM) when compared to concentrations measured *in vivo* during drug treatment.

The human COX-2 promoter is known to have two NFAT binding sites (Klein et al. 2007). Increased NFAT transcriptional activity can contribute to both carcinoma invasion and migration *in vitro* and it has been suggested that NFAT may promote tumor progression partly by up-regulating COX-2 (Buchholz and Ellenrieder 2007). There are several studies that indicate that the procarcinogenic role of COX-2 is mediated via NFAT. Increased COX-2 expression in colon carcinoma cells induced cell invasion *in vitro* and conversely, inhibition of NFAT reduced cell invasion (Corral et al. 2007). Activation of NFAT1 increased breast cancer cell line invasion in a COX-2 –dependent manner (Yiu and Toker 2006). Furthermore, NFAT is known to be essential for UV-induced COX-2 induction in keratinocytes (Flockhart et al. 2008).

2.2 Post-transcriptional regulation of COX-2 expression and PGE_2 production

Regulation of mRNA stability and translation have been shown to be central in the regulation of COX-2 expression (Dixon et al. 2000). Exon 10 of the COX-2 gene encodes the whole 3'-untranslated region (3'-UTR) that contains several copies of mRNA instability elements. mRNA stability and translation of many transiently expressed cytokines and proto-oncogenes have been reported to be regulated by AUUUA motifs (AU-rich element, ARE) that are present within the 3'-UTRs. (Barreau et al. 2005)

AREs have been shown to regulate gene expression by interacting with different RNA-binding proteins (Dean et al. 2004). Several RNA-binding proteins have been shown to bind the 3'-UTR of COX-2 mRNA: AU-rich element/poly(U)-binding/degradation factor-1 (AUF1) (Lasa et al. 2000), β-catenin (Lee and Jeong 2006), Hu antigen R (HuR) (Dixon et al. 2001), tristetraprolin (TTP) (Sawaoka et al. 2003), T-cell intracellular antigen 1 (TIA-1) and TIA related protein (TIAR) (Cok et al. 2004). The function of the majority of these proteins is known. HuR and β-catenin have been reported to stabilize mRNAs (Dixon et al. 2001, Lee and Jeong 2006), TIA-1 and TIAR are reported to be translational silencers (Gueydan et al. 1999, Piecyk et al. 2000) and TTP is known to destabilize target mRNAs (Sawaoka et al. 2003). AUF1 may play a role in both the degradation and stabilization of target mRNAs (Guhaniyogi and Brewer 2001).

Various signaling pathways (including mitogen-activated protein kinases, AMP-activated protein kinase, and protein kinase C family) are involved in the regulation of COX-2 expression at the post-transcriptional level in a cell-type dependent manner (Eberhardt et al. 2007). These signaling pathways act by directly or indirectly regulating the activity, localization or expression of the RNA-binding proteins.

The stability of COX-2 mRNA has been shown to be regulated by various pathophysiological and pharmacological factors. Taxanes, angiotensin II and gastrin all seem to be able to increase COX-2 mRNA stability by a mechanism related to HuR (Subbaramaiah et al. 2003, Doller et al. 2008, Subramaniam et al. 2008). In addition, transforming growth factor β, glycogen synthase kinase-3β, IL-1β and Zn^{2+} (Sheng et al. 2000, Tamura et al. 2002a, Harding et al. 2006, Thiel et al. 2006, Wu et al. 2008) have been reported to increase COX-2 mRNA stability whereas dexamethasone and thalidomide were found to destabilize COX-2 mRNA (Ristimäki et al. 1996, Lasa et al. 2001, Jin et al. 2007).

One interesting new area in the post-transcriptional regulation of COX-2 expression is microRNAs (miRNAs). miRNAs are a novel family of small (~19-22 nt) noncoding RNAs transcribed by genomes of most metazoa. They differ in sequences but are known to be involved in sequence-specific posttranscriptional regulation by affecting mRNA stability and/or translation. (Nilsen 2007) During embryo implantation, two uterine miRNAs, mmu-miR-199a* and mmu-miR-101a interact with the 3'UTR of COX-2 mRNA in mouse uterus causing to its translational repression (Chakrabarty et al. 2007, Daikoku et al. 2008). In colon cancer cell lines, miR-101 has been shown to directly silence COX-2 through a translational mechanism (Strillacci et al. 2008).

2.3 Signaling pathways regulating COX-2 expression

A variety of factors have been reported to stimulate COX-2 expression. Proinflammatory factors (e.g. IL-1, TNF-α, IFN-γ, LPS, TPA), hormones (e.g. follicle-stimulating hormone, luteinizing hormone, estrogen), growth factors (e.g. EGF, PDGF, FGF) and oncogenes (e.g. v-Src, v-Ras) have been reported to induce COX-2 expression. (Tanabe and Tohnai 2002) LPS was the first inducer of COX-2 expression that was identified in macrophages (Lee et al. 1992). LPS binds to Toll-like-receptor-4 that is a transmembrane protein with an extracellular domain consisting of leucine-rich repeats and a cytoplasmic domain that is homologous to that of the IL-1 receptor (Rock et al. 1998). The signaling pathways of most common COX-2 inducers are shown in Figure 4.
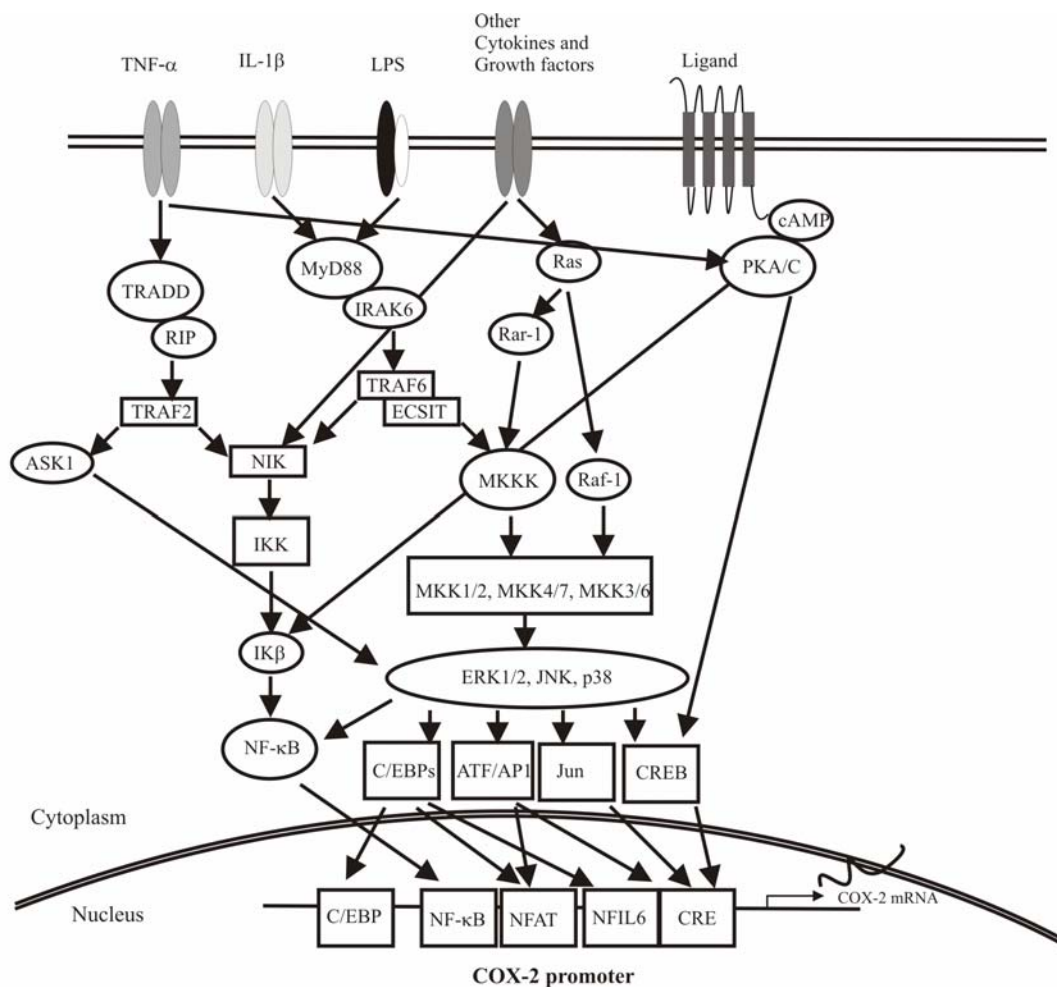
Figure 4. Most common signaling pathways involved in the transcriptional regulation of COX-2 expression. *TRADD=TNF receptor-1 associated via death domain, RIP=receptor interactive protein, death domain kinase, TRAF=TNF receptor-associated factor, ASK=apoptosis signal-regulating kinase, NIK=NF-κB-inducing kinase, IKK=IκB kinase, NF-κB=nuclear factor kappa-light-chain-enhancer of activated B cells, MyD88=myeloid differentiation factor 88, IRAK=IL-1 receptor-activated kinase, ECSIT=evolutionarily conserved signaling intermediate in Toll pathways, MKKK=MAPK kinase kinase, MKK=MAPK kinase, Erk1/2=Extracellular signal-regulated kinase 1 and 2, JNK= c-Jun N-terminal kinase, cAMP=cyclic adenosine monophosphate, PKA/C=protein kinase A/C, C/EBP=ccaat enhancer binding protein, ATF=activator transcription factor, AP1=activator protein-1, CREB=cAMP response element binding, NFAT=nuclear factor of activated T-cells, NFIL-6=nuclear factor interleukin-6, CRE=cAMP response element. (Modified from Tanabe and Tohnai 2002)*

A clinically interesting and important route of COX-2 mRNA and protein expression is the cAMP-dependent signaling pathway. Various studies have shown that activation of cAMP pathway may increase the level of COX-2 mRNA and/or protein expression. (Klein et al. 2007) Klein and co-workers concluded that cAMP acts as a positive modulator of COX-2 expression (Klein et al. 2007).

A serine-threonine kinase pathway, PI3K/Akt, has been shown to be a mediator of UVB-induced COX-2 expression in human keratinocytes (Tang et al. 2001, Takeda et al. 2004). PI3K-inhibitor studies in the endometrial cancer cell line, RL 95-2 demonstrated the inhibition of IκB phosphorylation, reduced NF-κB nuclear activity and decreased COX-2 expression, suggesting that PI3K/Akt regulates COX-2 expression via activation of NF-κB-mediated signaling (St-Germain et al. 2004).

Another serine-threonine kinase pathway, mitogen-activated protein kinases (MAPKs) has also been reported to regulate COX-2 expression (Tanabe and Tohnai 2002). Proinflammatory signals are mediated in the cell via activation of one or more members of this kinase family (Su and Karin 1996). Pharmacological inhibition or dominant-negative knockout of the MAPKs has been shown to reduce COX-2 induction and subsequent PGE_2 production (Guan et al. 1998, Molina-Holgado et al. 2000). The MAP kinase pathways are discussed in more detail in chapter 3.

Activator protein (AP)-1 is a transcription factor complex that is formed from heterodimers of Fos and Jun or homodimers of Jun proteins. MAPKs have been shown to directly phosphorylate proteins of the AP-1 complex and thus modulate AP-1 activity (Karin 1995). AP-1 binds to the CRE sequence and thus activates the expression of the COX-2 gene (Tanabe and Tohnai 2002).

In addition to serine-threonine kinases, also cytosolic tyrosine kinases protein kinase C (PKC) and IκB kinases (IKKs) can influence COX-2 expression (Huang et al. 2003). Tumor necrosis factor (TNF)-α has been shown to activate

phospholipase C-γ2 and subsequently PKC-α and protein tyrosine kinase, which activated NF-κB via NF-κB-inducing kinase and IKK1/2 that led to induction of COX-2 expression in human lung epithelial cells (Chen et al. 2000). Inhibitors of tyrosine kinases have been reported to inhibit IL-1β –induced COX-2 expression (Akarasereenont et al. 1994).

2.4 Pharmacological regulation of COX-2 expression and PGE$_2$ production

The application of willow tree bark for stiff and painful joints was recommended in the Ebers papyrus, which is the first known reference to the anti-inflammatory action of salicylates in 1534 BC. In 1828 salacin (named after its source, *Salix alba*; the white willow) was first isolated from willow bark and in 1874 acetylsalicylic acid was produced synthetically on an industrial scale (Hawkey 2005).

While the analgesic, antipyretic and anti-inflammatory properties of acetylsalicylic acid have been known since the end of the 19th century, its mechanism of action remained a mystery until the 1970s. In 1971, Sir John Vane showed that acetylsalicylic acid (aspirin)-like compounds act by inhibiting prostaglandin production (Vane 1971). This major discovery was awarded the Nobel Prize in Physiology and Medicine that Vane shared with Bergström and Samuelson in 1982.

NSAIDs can be grouped according to their chemical structure, i.e. salicylates, arylalcanoid acids (diclofenac, indomethacin, nabumetone, sulindac), 2-aryl-proprionic acids or profens (ibuprofen, flurbiprofen, ketoprofen, naproxen), N-arylanthranilic acids or fenamic acids (mefenamic acid, meclofenamic acid), pyrazolidine derivatives (phenylbutazone), oxicams (piroxicam, meloxicam), sulfonanilides (nimesulide) and coxibs (celecoxib, etoricoxib, lumiracoxib, rofecoxib, valdecoxib) (Figure 5). Nimesulide and coxibs are considered as COX-2 selective drugs. As a group, though, the NSAIDs are structurally different and differ in pharmacokinetic properties, but ultimately the mechanism

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of action, inhibition of prostaglandin synthesis, is very similar for all members of the NSAID group of drugs. (Flower et al. 1972, Hinz et al. 2007)
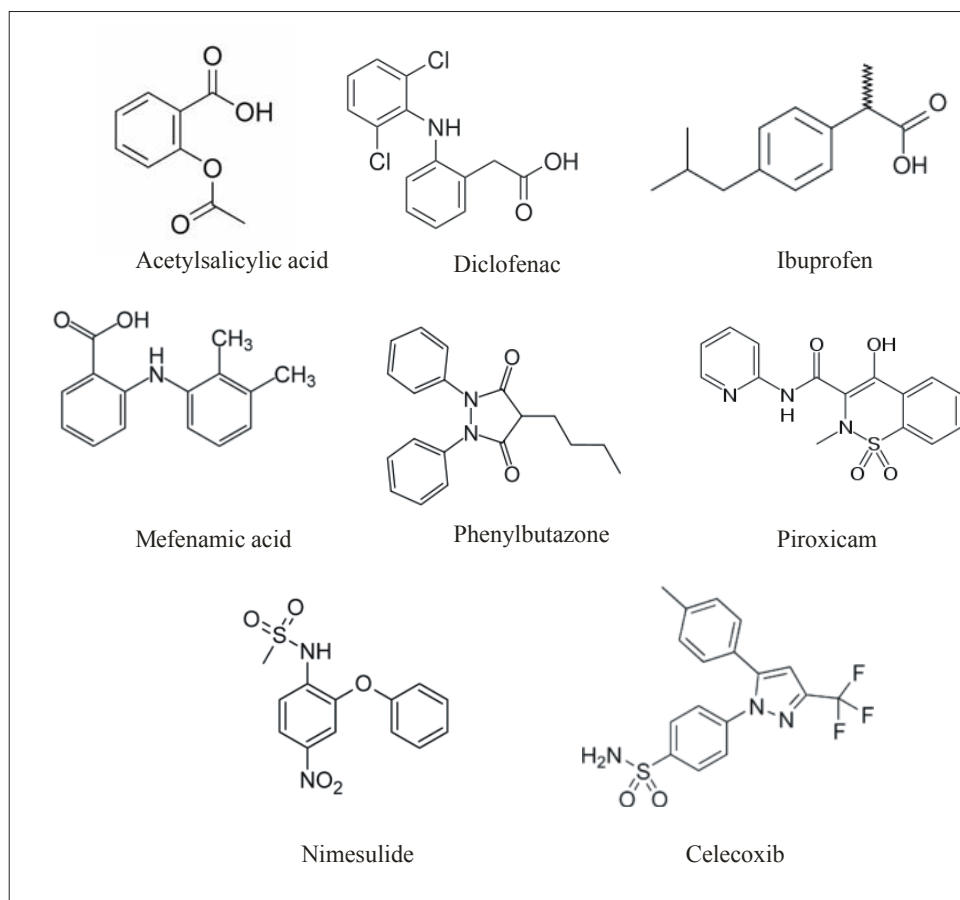
Figure 5. *Chemical structures of some NSAIDs*

The efficacy and toxicity of NSAIDs is mainly due to inhibition of COX enzymes. COX-1 is a constitutive enzyme responsible for housekeeping functions in organs including stomach, kidney, intestine and platelets, whereas COX-2 is an inducible enzyme exerting its action at inflammatory sites. Traditional NSAIDs inhibit both COX-1 and COX-2 enzymes. Selective inhibitors of COX-2 were developed on the basis of the theory that COX-1 mediates the biosynthesis of physiological prostanoids that regulate vascular tone and mucosal integrity, whereas COX-2 is the inducible isoform responsible for PG production in inflammation. (Hinz et al. 2007) In 1995, the first generation COX-2 selective NSAIDs, celecoxib and rofecoxib, entered clinical trials (Hawkey 2005). As predicted from biological experiments and animal models,

specific blockade of the COX-2 enzyme had a therapeutic analgesic effect which was associated with a lower risk of gastrointestinal complications, primarily perforations, ulcers and bleedings compared to "old" non-selective COX inhibitors (Wolfe et al. 2002).

The VIGOR study, which compared rofecoxib and naproxen in gastrointestinal events, raised concern about COX-2 selective NSAIDs in particular, the putative increased cardiovascular risk (Bombardier et al. 2000). The APPROVE study (adenomatous polyposis prevention study with rofecoxib) confirmed the concern (Bresalier et al. 2005). In 2004, rofecoxib was withdrawn from the global market (Hawkey 2005). It was suggested that inhibition of COX-2 –dependent synthesis of vasoprotective prostacyclin in endothelial cells, while leaving the platelet COX-1 –derived formation of prothrombotic thromboxanes unaltered, results in an imbalance between the eicosanoids that leads to thrombotic reactions (Flavahan 2007). This may not be the only mechanism for the cardiovascular risk of COX-2 inhibitors. The adenoma prevention with celecoxib (APC) –study showed that acetylsalicylic acid administration did not abolish the potential cardiovascular injury of celecoxib (Solomon et al. 2005). The TARGET trial compared lumiracoxib, the most selective COX-2 inhibitor so far, with naproxen and ibuprofen and found no correlation between the incidence of myocardial infarction and treatment with lumiracoxib (Farkouh et al. 2004). Further, the MEDAL study compared etoricoxib and diclofenac in 34 701 patients with OA or RA and the results showed that rates of thrombotic cardiovascular events were similar in both treatment groups (Cannon et al. 2006). Kearney and co-workers did a meta-analysis of randomized trials comparing COX-2 selective inhibitors with traditional NSAIDs and concluded that selective COX-2 inhibitors are associated with a moderately increased risk of vascular events but that high dose regimens of some traditional NSAIDs are also associated with a similar excess risk (Kearney et al. 2006). A good explanation for the cardiovascular events of both NSAIDs and COX-2 inhibitors is that COX-2 –derived prostaglandins upregulate thrombomodulin (a thrombin inhibitor) expression in human smooth muscle cells and this leads to prothrombotic effects platelet-independently (Rabausch et al. 2005). Warner and Mitchell stressed that one property that is

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common for NSAIDs and COX-2 inhibitors is that they both inhibit COX-2. This led to the conclusion that if COX-2 inhibitors provoke thrombotic events, the same has to be true for the traditional NSAIDs. (Warner and Mitchell 2008)

An interesting new regulatory element on prostaglandin E_2 production is enzyme mPGES-1. Macrophages derived from mPGES-1 knockout mice do not produce PGE_2 in response to stimulation with LPS (Trebino et al. 2003, Samuelsson et al. 2007). COX-2 and mPGES-1 seem to be coupled – long-term NSAID treatment has been shown to decrease both COX-2 and mPGES-1 production in articular cartilage from osteoarthritis patients (Alvarez-Soria et al. 2008). A new advance in the field of prostaglandin research is the development of dual inhibitors of mPGES-1 and 5-LO that potently inhibit both enzymes with significantly less pronounced inhibition on COX-enzymes (Koeberle et al. 2008).

Anti-inflammatory steroids have been reported to regulate COX-2 expression and PGE_2 production in multitude of ways. Glucocorticoids have been shown to induce the synthesis of lipocortins that are inhibitors of PLA_2 which releases arachidonic acid from cell membrane phospholipids (Di Rosa et al. 1984). Glucocorticoids are also known to down-regulate COX-2 expression in both transcriptional and post-transcriptional manners. The glucocorticoid-mediated suppression of COX-2 gene has been reported to be mediated by AP-1 and NF-κB transcription factors (Yang et al. 1990, Auphan et al. 1995). Anti-inflammatory steroid dexamethasone has been shown to act also at the post-transcriptional level by destabilizing COX-2 mRNA (Ristimäki et al. 1996, Lasa et al. 2001). Glucocorticoids control PGE_2 production also at the level of mPGES-1. Dexamethasone has been reported to completely suppress the inducible effect of proinflammatory cytokines on mPGES-1 expression (Thorén and Jacobsson 2000).

3. Mitogen-activated protein kinases

MAPKs are important signal transduction pathways in inflammation and have also been shown to participate in a variety of cellular processes, including cell differentiation, proliferation, movement and apoptosis. (Su and Karin 1996, Turjanski et al. 2007)

3.1 MAPK signaling pathways

The MAPK signaling cascade consists of evolutionary conserved enzymes that connect cell-surface receptors to regulatory targets within cells. The MAPKs are a family of serine/threonine kinases that are activated by dual phosphorylation at the tripeptide motif, Thr-X-Tyr, in response to extracellular stimuli. MAPK signaling pathways include extracellular signal-regulated kinase 1 and 2 (Erk1/2), p38 and c-Jun N-terminal kinase (JNK). The sequence of the tripeptide motif is unique to each specific MAPKs: Erk (Thr-Glu-Tyr), p38 (Thr-Gly-Tyr) and JNK (Thr-Pro-Tyr). (Su and Karin 1996, Dong et al. 2002)

The activation of the MAPK signaling pathway is due to a process where MAPK kinase kinase (MKKK or MEKK) phosphorylates and activates the next member, MAPK kinase (MKK or MEK). MKK is a dual-specificity protein kinase that leads the process forward: the phosphorylation of both threonine and tyrosine residues of the Thr-X-Tyr motif and the activation of the MAPK itself. (Chang and Karin 2001) Once activated, MAPKs phosphorylate target substrates on serine or threonine residues that are followed by a proline. The physiological substrates contain specific interaction motifs that define the substrate selectivity of different MAPKs. A schematic presentation of MAP kinase pathways is shown in Figure 6.
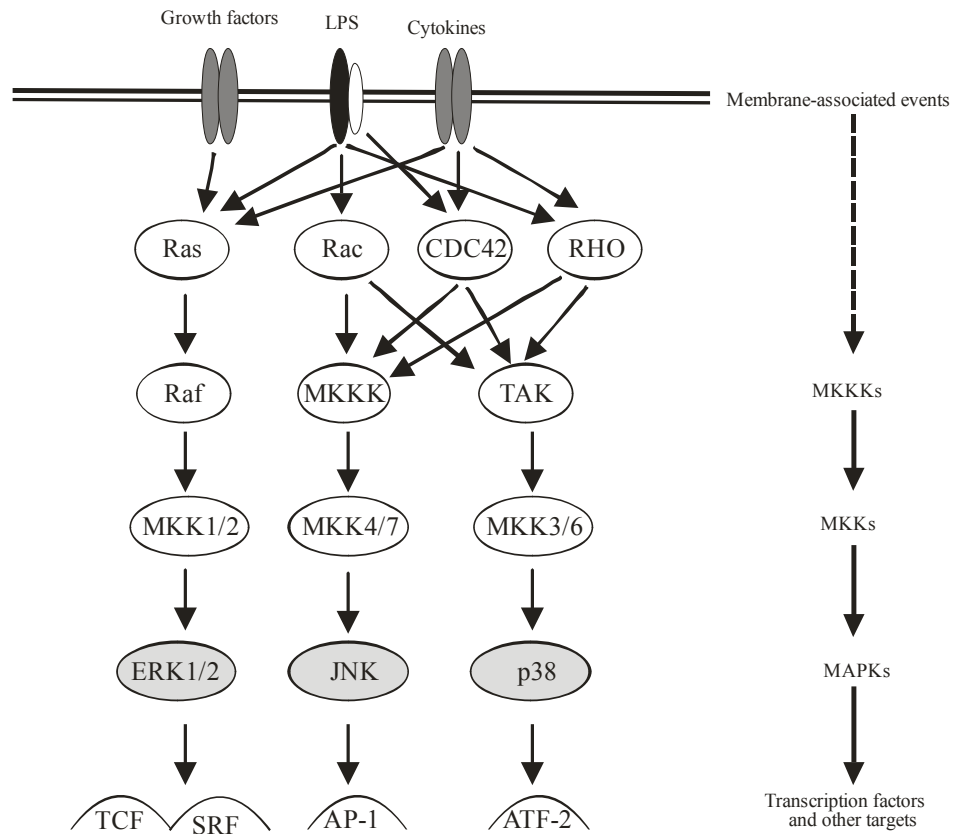
Figure 6. *A schematic figure of MAPK signaling pathways. Ras=G protein encoding oncogene, Raf=serine/threonine kinase, MKK1/2=MAPK/ERK kinase 1 and 2, ERK1/2=extracellular signal-regulated kinase 1 and 2, TCF=ternary complex factors, SRF=serum response factor, Rac=a member of the Rho family of Ras-related proteins, MKKK=MAPK kinase kinase, MKK4/7=MAPK kinase 4 and 7, JNK=c-Jun N-terminal kinase, AP-1=activator protein-1, CDC42=cell division cycle 42, RHO=GTP-binding protein of Ras-like proteins, TAK=Tumor growth factor-β activated kinase, MKK3/6=MAPK kinase 3 and 6, ATF-2=activating transcription factor-2*

3.2 p38 pathway

The p38 pathway is activated by several stimulants, including many inflammatory cytokines and cellular stress inducing factors like ultraviolet light. Also some normal physiological agents like hormones and ligands of G-protein coupled receptors and receptor tyrosine kinases can activate p38 cascade. (Martin-Blanco 2000)

The p38 pathway consists of several MKKKs, including MKK kinases (MKKKs)1-4, mitogen-activated protein triple kinase (MLTK)-2 and -3, apoptosis-signal regulating kinase (ASK)-1, tumor progression locus (TPL)-2

and TBF-β-activated kinase (TAK)-1. The MKKs include MKK3 and MKK6. In addition, MKK4, an upstream kinase of JNK, can activate p38 in response to certain stimuli in some cell types (Bobick and Kulyk 2008). Downstream of p38 itself is MAPK-activated protein kinase (MAPKAPK)-2. Most of the above members of MKKKs are not specific for the p38 pathway; they can phosphorylate members of other MAPK pathways as well. (Kyriakis and Avruch 1996, Kyriakis and Avruch 2001) The substrates of the p38 pathway include other kinases, cytosolic proteins and transcription factors (Saklatvala 2004).

There are four known p38 isoforms: the original isoform p38α, and p38β, p38γ and p38δ, which are all products of different genes. The human p38α MAPK was described as a molecular target of the pyridinyl imidazole inhibitors that were known to inhibit the production of IL-6 and TNF-α in LPS-treated human monocytes (Lee et al. 1994). Pyridinyl imidazole inhibitors were found to inhibit also p38β. p38α and p38β share also similar substrates like ATF-2 and MAPKAPK-2 (Zarubin and Han 2005). MKK3 is known to preferentially activate p38α and β whereas MKK6 activates all known p38 isoforms. (Roux and Blenis 2004) p38α differs from other isoforms in that it can be activated also by an MKK3/MKK6 –independent pathway. A noncatalytic scaffolding protein, TAK1-interacting protein (TAB1) can trigger the enzyme activity of p38α. (Ge et al. 2002) In addition to TAB1, Robidoux reported that in adipocytes, JNK interacting protein (JIP)-2 could create cell surface-linked complexes with MKK3 and p38α (Robidoux et al. 2005). Takaesu showed that in differentiating myocytes JNK-associated leucine zipper protein was able to activate both p38α and p38β (Takaesu et al. 2006). Of the four isoforms, p38α is the best characterized and according to current knowledge, it is the most physiologically relevant kinase in inflammation. The p38β gene shares >70% identity to p38α. Both p38α and β are expressed in several tissues. p38γ and p38δ share ~60% identity with p38α. p38γ is expressed largely in skeletal muscle, whereas p38δ is expressed in several adult tissues (i.e. lung, pancreas, small intestine, kidney, testis) as well as during development. (Kumar et al. 2003) Less is known about the role of p38γ and p38δ in inflammation.

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Knockout of the p38α gene (p38α$^{-/-}$ mice) leads to lethality by embryonic day 10; developing embryos exhibit faulty erythropoiesis and placental defects (Adams et al. 2000, Tamura et al. 2000). However, heterozygous mice (p38α$^{+/-}$) which display decreased p38α expression and activity have a normal phenotype. (Tamura et al. 2000, Otsu et al. 2003) p38β knockout mice are viable and exhibit a normal phenotype. In addition, they display normal stress-activated signaling in primary mouse embryonic fibroblasts as well as normal T-cell development and responses to proinflammatory stimuli LPS and TNF in their immune systems. (Beardmore et al. 2005) Mice lacking p38γ or p38δ are fertile and phenotypically normal if housed under specific pathogen- and stress-free conditions. Similarly, the mice lacking both p38γ and p38δ genes have normal phenotype. (Sabio et al. 2005)

p38 plays a critical role in the intracellular signaling cascades of various immune and inflammatory responses. p38 kinase can regulate gene expression by at least four different important routes. Firstly, p38 is able to regulate transcription of target genes via phosphorylation of transcription factors. Secondly, p38 can regulate the stability of mRNAs via downstream kinases. Thirdly, p38 is able to regulate mRNA translation into protein by the downstream kinases which phosphorylate AU-binding proteins that control translation. Fourthly, p38 can regulate the phosphorylation of histone H3 in chromatin at NF-κB binding sites in certain genes.

p38 has been shown to regulate chemokine expression and the level of cellular chemotactic responses. Inhibition of p38 pathway has been shown to decrease MCP (monocyte chemoattractant protein)-1, MIP (macrophage inflammatory protein)-1α/β and MIP-2 expression and the cellular responses of MCP-1, MIP-1α and RANTES (regulated upon activation, normal T cell expressed and secreted) (Schieven 2005). Lee et al. showed that inhibition of p38 led to decreased TNF-α production, and IL-12 production was shown to be reduced in MKK3 knockout mice (Lee et al. 1994, Lu et al. 1999). Interestingly, LPS-induced production of IL-1β, IL-6, IFN-γ and nitric oxide was markedly reduced in MAPKAPK-2 knockout mice and this resembled the effect of treatment with

the p38 inhibitor, SB203580 (Kotlyarov et al. 1999). In chondrocytes, monosodium urate has been shown to activate MMP-3 and iNOS expression in a p38-dependent manner (Liu et al. 2004). Recently, Lin and co-workers reported that the effect of glucosamine on MMP-3 could be mediated by p38 (Lin et al. 2008a).

One interesting role for p38 is the regulation of mRNA stability and mRNA translation of inflammatory proteins through ARE binding proteins in the 3'-UTR of the mRNAs (Winzen et al. 1999, Clark et al. 2003). TNF-α is an important example of a gene involved in p38-mediated mRNA stabilization. Regulation of TNF-α mRNA stability by the p38 pathway depends on the cell system being used (Clark et al. 2003). p38 is reported to regulate mRNA stability and mRNA translation by phosphorylating mRNA-binding proteins like TTP through MAPKAPK-2 and eukaryotic elongation factor 2 kinase (Knebel et al. 2001, Hitti et al. 2006).

p38 may also be involved in chondrogenesis. Stanton and co-workers have reported that the prechondrogenic mesenchymal cells of the mouse embryo limb bud express all p38 isoforms (Stanton et al. 2004). In addition, endogenous p38 activation and spontaneous chondrogenesis have been shown in embryonic chick limb bud mesenchyme cell micromass cultures (Oh et al. 2000). When constitutively active, MKK6 was transfected to prechondrogenic limb mesenchyme and a clear enhancement in the expression of SOX9 (SRY box containing gene 9) was seen (Weston et al. 2002).

3.3 JNK pathway

Most stimuli that activate p38 pathway activate also the JNK pathway. Principally, inflammatory cytokines and environmental stress can activate JNK cascade. Interestingly, also protein synthesis inhibitors, like cycloheximide and anicomycin can activate JNK pathway. (Kyriakis and Avruch 2001)

The MKKKs upstream of JNK are MKKK1-4, mixed lineage kinases (MLKs) 2-3, Tpl-2, dual leucine zipper-bearing kinase (DLK), TAO1-2, TAK1 and ASK1-2. These kinases phosphorylate the two kinases that activate JNK pathway: MKK4 and MKK 7. (Davis 2000, Kyriakis and Avruch 2001) TAK1 has been reported to be important in JNK activation mediated by inflammatory cytokines, toll-like receptors (TLR)-3, -4 and -9, and B and T cell receptors (Sato et al. 2005, Shim et al. 2005, Wan et al. 2006). MKKK3 seems to be the target for JNK activation by TLR-8 (Qin et al. 2006) and TPL2 and MLK3 have been reported to have a role in TNF-α –induced JNK activation (Brancho et al. 2005, Das et al. 2005). MKKK1, MLK2-3 and DLK specifically target and phosphorylate MKK4 and MKK7, the other MKKKs can activate also other pathways. Both MKK4 and MKK7 can activate JNK pathway by dual phoshorylation at Thr-Pro-Tyr motif, but MKK4 seems to primarily activate Tyr^{185} and MKK7 Thr^{183} (Gerwins et al. 1997, Lisnock et al. 2000). In addition to protein kinases, also scaffold proteins e.g. JNK-interacting proteins (JIPs) can activate and potentiate the JNK cascade (Morrison and Davis 2003).

Three JNK genes are known; i.e. JNK1, JNK2 and JNK3. The first two are expressed ubiquitously whereas JNK3 is more limitedly expressed and is restricted to brain, heart and testis. (Pulverer et al. 1991, Derijard et al. 1994, Kyriakis et al. 1994, Yang et al. 1997, Johnson and Nakamura 2007) Alternative splicing and exon usage means that there are multiple isoforms of JNK enzymes. These distinct forms of each JNK1, JNK2 and JNK3 seem to differ in their competence to bind and activate various substrate proteins. (Kallunki et al. 1994, Gupta et al. 1996) Alternative splicing in JNK proteins results in up to 10 different protein products that vary in size from 46 kDa to 55 kDa. Many studies have shown that the JNK1 gene produces a 46 kDa protein, the JNK2 gene a 55 kDa protein and the JNK3 gene 48 and 57 kDa proteins. The sequences of these different protein products show >80% homology. (Bogoyevitch 2006)

Like p38 kinase, also JNK has a role as a regulator of immune and inflammatory signals through the phosphorylation cascade. Firstly, the main substrate tightly controlled by JNK is the c-Jun gene. c-Jun proteins that are already present in the

cell are phosphorylated by JNKs in response to extracellular stimuli (Pulverer et al. 1991). c-Jun phosphorylation on Ser63 and Ser73 leads to improved c-Jun-dependent transcription. Secondly, a major target of JNK pathway is transcription factor AP-1 that is activated, in part, by phosphorylation of c-Jun and related molecules (Weston and Davis 2002). Thirdly, in addition to c-Jun, JNK phosphorylates also other AP-1 proteins, like JunB, JunD and ATF-2 (Davis 2000). Furthermore, several other transcription factors have been shown to be phosphorylated by JNKs, like NF-ATc1, HSF-1 and STAT-3 (Kyriakis and Avruch 2001).

JNK has also been shown to regulate the expression of inflammatory and other proteins by stabilizing mRNAs of CLMP, iNOS, IL-2, IL-3 and VEGF (Chen et al. 1998, Ming et al. 1998, Pages et al. 2000, Lahti et al. 2003, Sze et al. 2008). The stabilizing effect of JNK on CLMP and iNOS mRNA may be mediated by an RNA binding protein TTP (Korhonen et al. 2007, Sze et al. 2008). In terms of IL-2, the effects of JNK were reported to be regulated via the JNK-responsive element (JRE) in the 5'-UTR of IL-2 mRNA. Two RNA-binding proteins, Y box-binding protein and nucleolin, were found to recognize this element. (Chen et al. 2000)

Deficiency of JNK1, JNK2 or JNK3 does not lead to lethality or even to any clear defects. Embryos lacking JNK1 (JNK1$^{-/-}$) or JNK2 (JNK2$^{-/-}$) survive but, JNK1$^{-/-}$ and JNK2$^{-/-}$ double mutants die at mid-gestation due to disrupted apoptotic programmes in specific regions of the brain and defective neural tube closure (Kuan et al. 1999, Sabapathy et al. 1999). Mice lacking JNK3 that is the form predominantly expressed in the nervous system, show normal structural and cellular organization of brains. Functionally, JNK3$^{-/-}$ mice seem to have protection against an epileptogenic substance, kainic acid. JNK3$^{-/-}$ mice showed lower c-Jun phosphorylation and transcriptional activation and less hippocampal cell loss compared to wild type animals. (Yang et al. 1997) Interestingly, neither JNK1$^{-/-}$ nor JNK2$^{-/-}$ mice were protected against kainic acid but mice expressing a mutant form of c-Jun (c-Jun$^{A63/A73}$) lacking the JNK phosphoacceptor sites, showed the same phenotype as JNK3$^{-/-}$ mice (Behrens et al. 1999).

The role of JNK in apoptosis is well documentated (Davis 2000, Weston and Davis 2002, Hasala et al. 2007b) but the mechanism of JNK in apoptosis is still under debate and seems to depend on stimulus and cell type (Liu and Lin 2005). Two antihistamines, diphenhydramine and chlorpheniramine, have been shown to enhance apoptosis in human eosinophils in a JNK mediated manner (Hasala et al. 2007a). Chang et al. showed that TNF-α -mediated JNK activation accelerates the loss of the NFκB-induced antiapoptotic protein, c-FLIP, that is an inhibitor of caspase-8 (Chang et al. 2006). TNF-α induced JNK activation is known to be two-phased. The first phase (30 min) is intense and transient and is followed by the second more sustained phase that lasts many hours. Ventura and colleagues reported that the early phase of JNK activation could mediate cell survival, while the later and more sustained phase of activation mediated proapoptotic signaling. (Ventura et al. 2006) A histone H2A variant, H2AX that is phosphorylated in apoptotic cells has been shown to be a JNK substrate. H2AX phosphorylation is necessary for DNA ladder formation and the JNK/H2AX pathway cooperates with the caspase3/caspase-activated DNase pathway resulting in cellular apoptosis. (Lu et al. 2006, Sluss and Davis 2006)

There is no evidence to support an involvement of JNK pathway in chondrogenic differentiation within embryonic mesenchymal limb. Unlike Erk and p38, JNK phosphorylation cannot be detected in embryonic chick wing bud mesenchymal cells within 5-day period (Oh et al. 2000).

3.4 ERK1/2 pathway

ERK1 and ERK2 (also referred as p44/p42 MAP kinases) were the first identified and cloned MAPK (Sturgill et al. 1988, Ahn et al. 1991, Boulton et al. 1991). ERK1/2 pathway is activated by growth factors, cytokines, viral infection, transforming agents and carcinogens (Johnson and Lapadat 2002). ERK1 and ERK2 share 83% amino acid identity and are expressed to various extents in all tissues.

ERK1/2 pathway consists of the MKKKs A-Raf, B-Raf, Raf-1 (Kyriakis et al. 1992, Moodie et al. 1993) and c-Mos (Pham et al. 1995), and the MKKs MAPK/ERK kinases (MKK)1/2. MKKs are encoded by different genes but are very similar in terms of sequence, substrate specificity and regulation. (Zheng and Guan 1993b). ERK1/2 activation results in phosphorylation of various substrates in all cellular compartments including several membrane proteins (CD120a, Syk and calnexin), nuclear substrates (SRC-1, Pax6, NF-AT, Elk-1, MEF2, c-Fos, c-Myc and STAT3) and cytoskeletal proteins (neurofilaments and paxillin) (Chen and Cobb 2001).

The mammalian ERK MAPK pathway is involved in many physiological processes, including cell proliferation, differentiation and survival (Pearson et al. 2001). The activation of ERK1/2 induces proliferative signals that may contribute to normal and cancerous cell growth (Cowley et al. 1994). Consistently, in many human cancer types, abnormal activation of the ERK pathway, which is often due to mutation in the genes encoding molecules regulating ERK1/2, like Ras, is a common event (Hoshino et al. 1999). Interestingly, gene ablation studies of ERK1 and ERK2 have exposed that ERK2 knockout in mice leads to embryonic lethality whereas ERK-1 knockout mice are viable (Pages et al. 1999, Saba-El-Leil et al. 2003).

Given the prominent role of ERK1/2 signaling in the regulation of diverse parameters of cellular metabolism, it is not surprising that the spatial expression of active phosphorylated ERK1/2 is widely distributed at early stages of organogenesis in vertebrate embryos (Corson et al. 2003). The roles and patterns of ERK signaling are believed to be substantially altered during the developmental time course of chondrogenesis and can even vary according to anatomical location (Bobick et al. 2007).

3.5 Inhibition of MAPK

MAPKs are known to regulate the expression of cytokines and other mediators that are part of the pathogenic processes in inflammation. MAPKs maintain a

role not only in inflammation, but also in cellular-adaptive responses like apoptosis, regulation of cell-cycle and proliferation and development and differentiation. Pre-clinical studies with MAPK inhibitors have shown significant efficacy in many disease models, e.g. arthritis, pain, psoriasis, airway diseases and inflammatory bowel disease. Unfortunately, in clinical studies, most of the novel compounds have encountered problems related to toxicity and limited efficacy.

3.5.1 p38 inhibitors

p38 MAPK was originally discovered in a study into the effects of pyridinyl-imidazole compounds on IL-6 and TNF-α production in human monocytes (Lee et al. 1994). The 38 kDa proteins characterized were first called "cytokine-suppressive anti-inflammatory drug binding protein-1/2", later they were shown to exhibit protein kinase activity and named p38α and β (Han et al. 1994, Lee et al. 1994). SB203580, a classical p38 inhibitor, is a representative of pyridinyl-imidazole compounds (Cuenda et al. 1995). Several compounds in the pyrinidyl-imidazole group inhibit phosphorylated p38 by binding to the ATP site (Young et al. 1997). These compounds have been useful in the discovery of p38 and in *in vitro* studies but they were not suitable for oral drug delivery (Adams et al. 2001).

VX745 is a p38 inhibitor generated after pyridinyl-imidazoles (Fitzgerald et al. 2003). The selectivity of VX745 is based on the presence of Gly at residue 110. This is atypical as compared to other kinases and may well account for increased p38 selectivity.

BIRB796 is characteristic of diaryl urea compounds of p38 inhibitors (Pargellis et al. 2002). The mechanism of action of BIRB796 differs from previous compounds; BIRB796 acts by causing a malfunction in the activation loop of p38 and inhibiting the activation of p38 by MKK6 (Sullivan et al. 2005).

The importance of p38 pathway in the regulation of many inflammatory mediators makes it very interesting drug target. Several p38 inhibitors have got into clinical trials, some of them have been unsuccessful but there are reports of favourable responses in animal models and clinical studies (Dominguez et al. 2005, Goldstein and Gabriel 2005, Lee and Dominguez 2005).

Interestingly, experimental RA models have shown that p38 inhibitors are effective in reducing symptoms, paw swelling, inflammation, cartilage breakdown and bone erosion in the rat streptococcal cell wall arthritis model, the collagen induced arthritis –model in mice and the adjuvant and collagen induced arthritis –model in rats (Badger et al. 2000, Mclay et al. 2001, Nishikawa et al. 2003, Mbalaviele et al. 2006, Medicherla et al. 2006). In clinical studies, a large number of p38 inhibitors have been tested and found to be safe and effective in the reduction of IL-6, IL-8 and TNF-α production, C-reactive protein release in serum, and fever caused by endotoxin challenge in healthy volunteers (Fijen et al. 2001, Branger et al. 2002, Fijen et al. 2002, Parasrampuria et al. 2003, Schreiber et al. 2006). However, it seems that efficacy and safety of these compounds in RA patients is no better to placebo (Sweeney and Firestein 2006).

In Crohn's disease, BIRB796 failed to exert any a clinical benefits (Schreiber et al. 2006) whereas treatment with CNI1493, a non-selective p38 and JNK inhibitor led to a statistically significant improvement in the Crohn's Disease Activity Index without causing any serious side effects (Hommes et al. 2002).

3.5.2 JNK inhibitors

An anthrapyrazolone JNK inhibitor, SP600125, was reported in late 2001. SP600125 was shown to be highly selective towards JNK (>300-fold selectivity over p38 and ERK and between 10 to 100-fold selectivity over 14 other kinases tested). SP600125 is a reversible ATP-competitor that inhibits JNK mediated c-Jun phosphorylation. (Bennett et al. 2001) SP600125 was the first chemical inhibitor of JNK and it has been used in more than 1200 publications to reveal the importance of JNK pathway.

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CC-401 is a second generation JNK inhibitor that inhibits JNK signaling by competitive binding to adenosine triphosphate-binding site in the active, phosphorylated, form of JNK, resulting in inhibition of the phosphorylation of JNK targets. CC-401 is a potent inhibitor of all three forms of JNK and has at least 40-fold selectivity for JNK compared to other related kinases, like p38, ERK1/2, inhibitor of κB kinase (IKK2), PKC and zeta-associated protein of 70 kDa (ZAP70) (Uehara et al. 2004, Uehara et al. 2005). CC-401 has shown efficacy in a rat-model of immune-induced renal injury, and CC-401 and related compounds (CC0209766 or CC0223105) improved the survival rate in hepatic warm ischemia/reperfusion injury model from <40% to 60-100% (Uehara et al. 2005, Flanc et al. 2007).

In addition to SP600125 and CC-401, there are additional small molecule inhibitors of JNK that have largely been discovered in *in vitro* kinase assays against purified JNK. The small molecule JNK inhibitors include diaryl-imidazoles, (benzoylaminomethyl)thiopene sulfonamides, dihydro-pyrrolo-imidazoles, (benzothiazol-2-yl)acetonitrile, anilinoindazoles, anilino-bipyridines, pyridine carboxamides and anilino-pyrimidines (Scapin et al. 2003, Ruckle et al. 2004, Gaillard et al. 2005, Graczyk et al. 2005, Swahn et al. 2005, Liu et al. 2006, Swahn et al. 2006, Szczepankiewicz et al. 2006, Zhao et al. 2006, Liu et al. 2007). With respect to these compounds, AS601245 ((benzothiazol-2-yl)acetonitrile), has been tested in preclinical RA models and in models of cerebral and cardiac ischemia (Carboni et al. 2004, Ferrandi et al. 2004, Gaillard et al. 2005). In collagen-induced arthritis, AS601245 decreased paw swelling and reduced the clinical arthritis scores. Histologically cartilage erosion and synovial inflammation were decreased in AS601245-treated group. (Gaillard et al. 2005)

Some natural products have been shown to inhibit JNK. Latifolians A and B from an extract of New Guinea vine, *Gnetum latifolium*, were demonstrated to inhibit JNK3 *in vitro* (Rochfort et al. 2005).

Peptide inhibitors that can target or interrupt JNK signaling complexes have also been reported to inhibit JNK pathway. The JNK pathway uses JNK interacting protein (JIP) family scaffold proteins. It has been shown that overexpression of full length JIP1 or specific parts of the protein, like JNK binding domain (JBD), inhibit JNK activity. Since other MAPKs do not use JIPSs, it appears that the short JIP1 JBD derived peptides are rather selective and inhibit only JNK and its upstream activators, MKK4 and MKK7. (Borsello et al. 2003)

3.5.3 ERK1/2 inhibitors

To date, no direct ERK1/2 inhibitors have been described. The inhibitors of the upstream regulators, MKK1/2 and Raf, represent the most extensively studied approach for blocking ERK signaling. It is reasonable to inhibit MKK1/2 or Raf as a means to block ERK1/2 activation. MKK1/2 are the only known catalytic substrates of Raf kinases and for one's part, ERK1/2 is the only known substrate of MKK1/2. (Hoshino et al. 1999)

PD98059, PD198306 and U0126, the first ERK1/2 inhibitors available, target ERK1/2 by inhibiting the kinase function of MKK1/2 (Dudley et al. 1995, Favata et al. 1998). These compounds are highly selective but they lack the pharmaceutical properties needed to be successful clinical candidates (Davies et al. 2000). However PD98059 has proved useful in elucidating the role of ERK1/2 pathway in various cellular events, and it has been used in more than 7000 publications. The difference between MKK inhibitors and the majority of protein kinase inhibitors is that MKK inhibitors are non-ATP competitive.

CI-1040 (PD184352) is a highly selective and potent, and an orally active inhibitor of MKK1/2 and the first MKK1/2 inhibitor that entered clinical trials (Sebolt-Leopold et al. 1999). Unfortunately the results from phase II studies in patients with advanced non-small cell lung carcinoma, breast cancer, colorectal cancer and pancreatic cancer were negative (Rinehart et al. 2004).

The second generation of MKK1/2 inhibitors include PD0325901 and AZD6244 (ARRY-142886) (Sebolt-Leopold and Herrera 2004, Yeh et al. 2007). PD0325901 is a derivative of CI-1040 that is more than 50-fold more potent against MKK1/2 than the parent compound. It has also been reported to have improved bioavailability and longer duration of target inhibition compared to the original compound (Sebolt-Leopold and Herrera 2004). AZD6244 is an orally active benzimidazole derivative that is known to inhibit MKK1/2 *in vitro* (Yeh et al. 2007). Not only small molecule inhibitors, but also bacterial toxins have been reported to inhibit MKK function. Anthrax lethal factor, a protease from *Bacillus anthracis*, and Yersinia outer protein J have shown inhibitory potency against MKK1/2. (Orth et al. 1999, Bodart et al. 2002)

Since ERK1/2 MAPK is known to promote cell proliferation, cell survival and metastasis, the primary drug target of its inhibitors has been cancer. However, the MKK/ERK pathway has been shown to play a role in metalloproteinase production in a variety of cell types (Brogley et al. 1999, Brauchle et al. 2000). One possible mechanism for the role of ERK1/2 in inflammation is that ERK1/2 has as a downstream target Elk-1 that transactivates genes including transcription factor AP-1 that binds to the promoters of many cytokines and matrix metalloproteases (Wang et al. 2004). MKK1/2 activity has also shown to be up-regulated in joint tissue taken from RA patients (Schett et al. 2000, Thiel et al. 2007). Interestingly, an orally bioavailable MKK1/2 inhibitor, PD184352, was recently shown to prevent edema significantly, as well as clinical arthritis and histopathological changes in murine collagen-induced arthritis (Thiel et al. 2007).

There are several structurally different compounds that act as potential Raf kinase inhibitors (Smith et al. 2006). In addition to the small molecule inhibitors, Raf kinases can be inhibited by antisense inhibitors that block c-Raf-1 protein expression (ISIS-5132, a 20-base phosphorothioate DNA oligonucleotide) or by liposome-encapsulated antisense c-Raf-1 oligonucleotide (Gokhale et al. 2002).
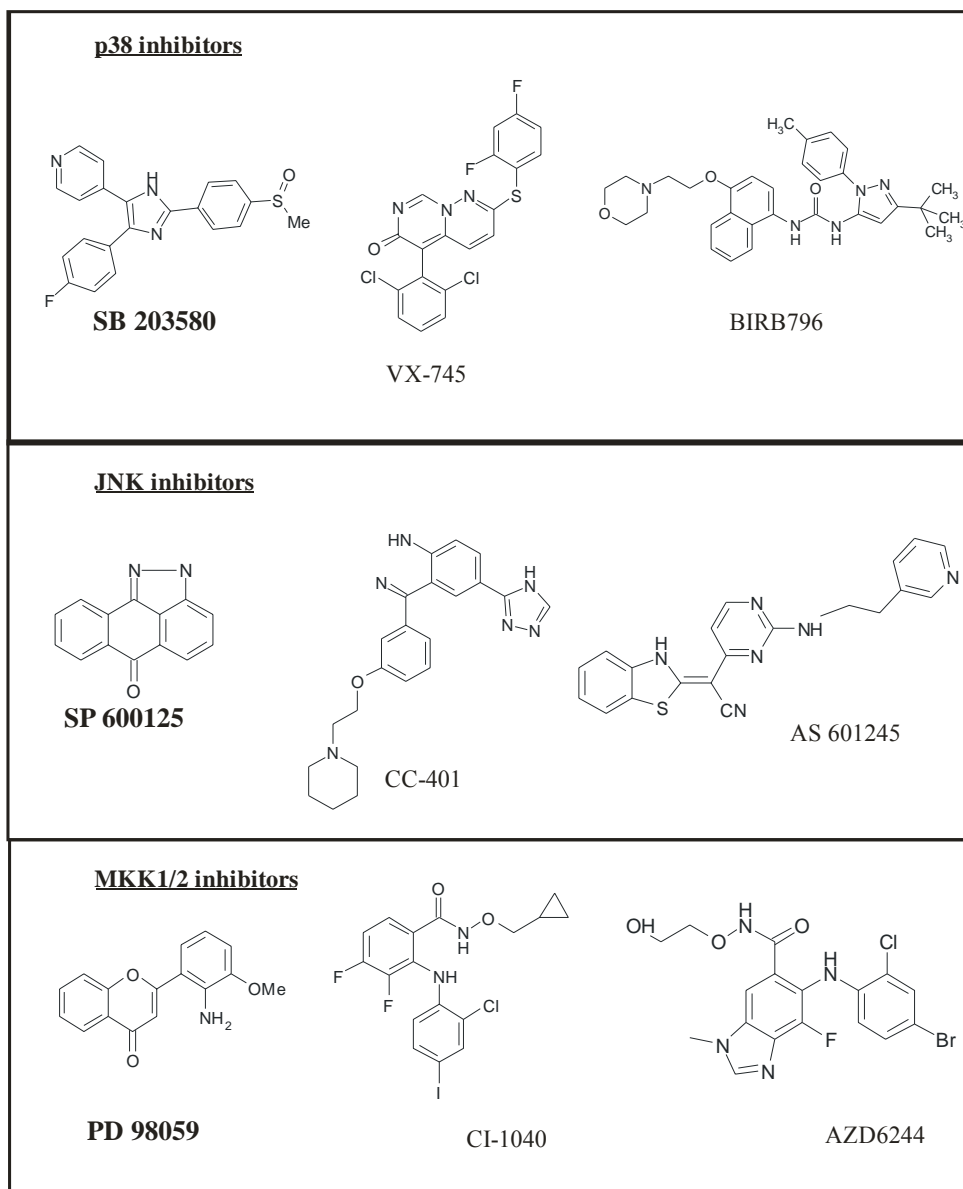
Figure 7. *Chemical structures of some MAPK inhibitors. The names of the inhibitors used in the present study are shown in bold.*

3.6 MAPK phosphatases

MAPKs have to be phosphorylated on both threonine and tyrosine residues for kinase activity. Both protein serine/threonine phosphatases and protein tyrosine phosphatases have been shown to be the negative regulators of MAPKs by dephosphorylating the kinases. The MAPK phosphatase (MKP) family consists of the serine/threonine phosphatases, tyrosine phosphatases and dual-specificity

phosphatases (DUSPs) which can dephosphorylate both phosphothreonine and phosphotyrosine residues on activated MAPKs. (Keyse 2000, Farooq and Zhou 2004) In human and mouse genome, at least 30 DUSP genes that share a common structure containing an N-terminal non-catalytic domain and a C-terminal catalytic domain have been identified (Muda et al. 1996a, Theodosiou and Ashworth 2002). Of these 30 phosphatases, 10 members have shown substrate specificity for MAPKs (Lang et al. 2006). Based on gene structure, substrate specificity for different MAPKs, and subcellular localization, these MKPs can be subdivided into three groups. Subgroup I consists of DUSP1/MKP-1, DUSP2/PAC-1, DUSP4/MKP-2 and DUSP5. The members of subgroup I are inducible nuclear enzymes. Subgroup II consists of cytoplasmic phosphatases that all primarily dephosphorylate ERK1/2: DUSP6/MKP-3, DUSP7/MKP-X and DUSP9/MKP-4. The members of the third group include DUSP8, DUSP10/MKP-5 and DUSP16/MKP-7 which all show substrate specificity towards p38 and JNK MAPKs. (Lang et al. 2006, Salojin and Oravecz 2007) Some basic properties of these 10 dual-specificity MKPs are shown in Table 2. The 20 remaining DUSPs are smaller proteins that are deficient in MAPK binding domain (MKB) in the presence of DUSP domain. Nonetheless, DUSP14/MKP6, DUSP3/VHR and DUSP22/VHX have been shown to function like MKPs (Marti et al. 2001, Alonso et al. 2002, Alonso et al. 2003)

It seems that MKPs differ from their substrate MAPKs in their expression profile. During development, depending on the cell-type or cellular activation, MKPs show regulated expression whereas MAPKs are expressed more commonly (Charles et al. 1993, Sun et al. 1993, Dickinson et al. 2002, Christie et al. 2005). MKPs have been demonstrated to possess substrate selectivity towards different MAPK isoforms. DUSP6/MKP-3 was the first MKP that was shown to inactivate only ERK1/2 *in vitro* and *in vivo* and to have only little or no activity towards JNK or p38 (Groom et al. 1996, Muda et al. 1996b). After that finding it has been shown that also other MKPs are able to inhibit p38, JNK or ERK1/2 altogether or with a degree of selectivity (Muda et al. 1996a, Tanoue et al. 1999, Slack et al. 2001, Tanoue et al. 2001). DUSP6/MKP-3 has been reported to recognize and bind ERK2 tightly with a conserved motif within the amino-

terminal non-catalytic domain. This phenomenon is followed by increased DUSP6/MKP-3 catalytic activity *in vitro*. (Camps et al. 1998, Muda et al. 1998) Catalytic activation of MKPs has been thought to be a general mechanism for MKPs'substrate selectivity (Camps et al. 2000).

Table 2. *Key properties for dual-specificity phosphatase (DUSPs) / MAP kinase phosphatases (MKPs)s. (Modified from Owens and Keyse 2001)*

Gene	MKP	Subcellular localization	Substrate preference
Subgroup I			
DUSP1	MKP-1	Nuclear	p38 ~JNK >> ERK1/2
DUSP2		Nuclear	ERK ~ p38 >> JNK
DUSP4	MKP-2	Nuclear	ERK ~ JNK >> p38
DUSP5		Nuclear	ERK
Subgroup II			
DUSP6	MKP-3	Cytoplasmic	ERK
DUSP7	MKP-X	Cytoplasmic	ERK
DUSP9	MKP-4	Cytoplasmic	ERK > p38
Subgroup III			
DUSP8		Cytoplasmic / nuclear	JNK, p38
DUSP10	MKP-5	Cytoplasmic / nuclear	JNK, p38
DUSP16	MKP-7	Cytoplasmic / nuclear	JNK, p38

3.6.1 MAPK phosphatase-1

MKP-1 was the first discovered MAPK selective phosphatase that was capable of dephosphorylating both phosphotyrosine and phosphothreonine residues (Sun et al. 1993). Mouse MKP-1 cDNA was first cloned in the screening of BALB/c 373 cDNA library and was recognized as an immediate-early transcript that is induced by growth factors (Lau and Nathans 1985, Charles et al. 1992). The homolog for the murine gene, named 3CH134, was soon identified also from human origin (named as CL100) and it was reported to be induced by oxidative stress (Keyse and Emslie 1992). The gene was found to code a 39.5 kDa protein that had (I/V)HCXAGXXR(S/T)AG (characteristic phosphatase catalytic site) in the C-terminus and a typical kinase-interaction domain in the N-terminus. It was named MAPK phosphatase (MKP)-1 because of its ability to dephosphorylate

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both phosphotyrosine and phosphothreonine residues. (Sun et al. 1993, Tamura et al. 2002b).

3CH134 and CL100 were first shown to be selective towards ERK1/2 *in vitro* (Alessi et al. 1993, Charles et al. 1993, Sun et al. 1993, Zheng and Guan 1993a). Subsequently MKP-1 was found to dephophorylate MAPKs in the order p38>JNK>>ERK1/2 (Franklin and Kraft 1997). MKP-1 is ubiquitously expressed and it can be highly up-regulated by stimuli like endotoxin, anisomycin, UV, p53, osmotic and heat shock, 12-O-tetradecanoylphorbol-13-acetate and growth factors (Tamura et al. 2002b). In human lymphoid organs, MKP-1 is constitutively expressed but one characteristictrant is that its expression is increased rapidly in response to cell activation. In LPS-stimulated murine macrophages, expression of MKP-1 rapidly increases and then returns to basal level in 2-3 hours (Chi et al. 2006).

Transcription factors AP-1 (c-Fos, c-Jun, ATF2), SP1, SP3, glucocorticoid receptor and NF-κB have been shown to bind to MKP-1 promoter and to induce transcription (Kwak et al. 1994, Laderoute et al. 1999, Ryser et al. 2004, Xu et al. 2004, Issa et al. 2007). Interestingly, phosphorylation and acetylation of histone H3 has been reported to change the chromatin at the MKP-1 gene locus, this being followed by an increase in both the association of RNA polymerase II to MKP-1 gene promoter and its transcription (Li et al. 2001).

In addition to transcriptional mechanisms, MKP-1 is also regulated at the post-transcriptional level. Interestingly, the mRNA of MKP-1 contains several evolutionally conserved AREs in the 3'-UTR (Charles et al. 1992, Emslie et al. 1994) and several RNA binding proteins have been reported to bind the MKP-1 mRNA and modulate its stability. TTP has been shown to destabilize MKP-1 mRNA in 3T3-L1 cells whereas NF90 and HuR have been reported to increase its stability in HeLa cells (Kuwano et al. 2008, Lin et al. 2008b). Interestingly, HuR has also been reported to increase MKP-1 mRNA translation, and NF90 is believed to suppress its translation (Kuwano et al. 2008).

MKP-1 is known to be an important negative regulatory participant in the inflammatory process. This observation led to a question about the effects of anti-inflammatory drugs on MKP-1 expression. MKP-1 expression was first shown to be increased in response to dexamethasone by Kassel and co-workers in mast cells (Kassel et al. 2001). The same phenomenon was demonstrated in RAW264.7 macrophages a few months later: Chen showed that in addition to dexamethasone, also CTB, a component of cholera toxin that is produced by the Gram negative bacterium *Vibrio cholerae,* was able to increase MKP-1 expression (Chen et al. 2002). In 1997 Swantek and co-workers showed that dexamethasone inhibited LPS-induced JNK activation (Swantek et al. 1997). Evidence for enhanced MKP-1 expression after dexamethasone-treatment supported this finding. The effect of dexamethasone on COX-2 expression was found to be mediated by decreased p38 activity and increased MKP-1 expression in HeLa cells (Lasa et al. 2000, Lasa et al. 2001, Lasa et al. 2002). Interestingly, Zhao and co-workers reported that the ability of synthetic corticosteroids to induce MKP-1 expression was comparable to the anti-inflammatory potencies of these drugs (Zhao et al. 2005). Abraham et al. showed that in bone marrow macrophages from MKP-1$^{-/-}$ mice, dexamethasone did not inhibit p38 and JNK activity. Similarly, the effect of dexamethasone on many inflammatory genes was decreased. (Abraham et al. 2006)

In 1996, the Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute generated mice, embryonic stem cells and mouse embryo fibroblasts that are deficient of the MKP-1. They reported that MKP-1$^{-/-}$ mice were born at a normal frequency and they were fertile and did not present any phenotypic or histologic abnormalities. (Dorfman et al. 1996) Later, it has been shown that although MKP-1$^{-/-}$ mice do not show any significant abnormalities in lymphoid or myeloid development, the deletion of MKP-1 leads to markedly elevated levels of proinflammatory cytokines and also the other inflammatory mediators in response to LPS stimulation compared to wild type animals. This feature is accompanied by a significally increased mortality rate in response to endotoxin. In a low-dose endotoxin challenge model, the strong immune response is mediated by the release of macrophage-derived cytokines TNF-α, IL-6, IL-12

and IL-10 and the proinflammatory chemokines CCL3, CCL4 and CXCL2 leading to LPS-induced hepatotoxicity and shock. (Chi et al. 2006, Hammer et al. 2006, Salojin et al. 2006, Zhao et al. 2006) MKP-1$^{-/-}$ mice have been shown to be more sensitive to the progression of septic shock syndrome, associated with hypotension, respiratory failure, increased nitric oxide production, and multiple organ failure which has appeared as renal, hepatic, and pulmonary damage after LPS-treatment (Zhao et al. 2006). MKP-1 is able to inhibit phosphorylation of p38 MAPK in different mouse and human cell types (Wang and Liu 2007). In studies using LPS-stimulated MKP$^{-/-}$ macrophages, MKP-1 has been shown to have only a limited effect on JNK phosphorylation (Hammer et al. 2006, Salojin et al. 2006). Interestingly, MKP-1 has been shown to be an important factor in preventing the development of autoimmunity, especially arthritis. Salojin and co-workers reported that after collagen immunization MKP-1$^{-/-}$ mice developed more severe arthritis than their wild type counterparts (Salojin et al. 2006).

4. Aurothiomalate

From the earliest civilizations, gold has been described as *rex metallorum* and symbolized with the sign of the sun meaning the origin of power, richness and benefits to life. The first notes of the medical use of gold can be found from Chinese texts 2500 BCE. Metallic gold power has been used in India to treat asthma and arthritis for at least two millennia. The history of aurothiomalate, gold sodium thiomalate, in the treatment of RA began in the 1930's when Dr. Forestier showed that it has anti-rheumatic properties (Forestier 1935).

4.1 Aurothiomalate as one of the DMARDs

Disease-modifying antirheumatic drugs (DMARDs) (including e.g. cyclosporin A, gold (aurothiomalate and auranofin), hydroxychloroquine, leflunomide, methotrexate, D-penicillamine, and sulfasalazine) can with early initiation lead to substantial improvement in symptoms of rheumatoid arthritis and retard radiographic progression (Tsakonas et al. 2000, Lard et al. 2001). These agents

share several common features, like slow onset of therapeutic effect and poorly understood molecular mechanism of action. Felson and co-workers published a meta-analysis of blinded clinical trials in 1990 and showed that the efficacy of aurothiomalate, methotrexate, D-penicillamine, and sulfasalazine are comparable and that efficacy of auranofin and hydroxychloroquine are somewhat weaker (Felson et al. 1990).
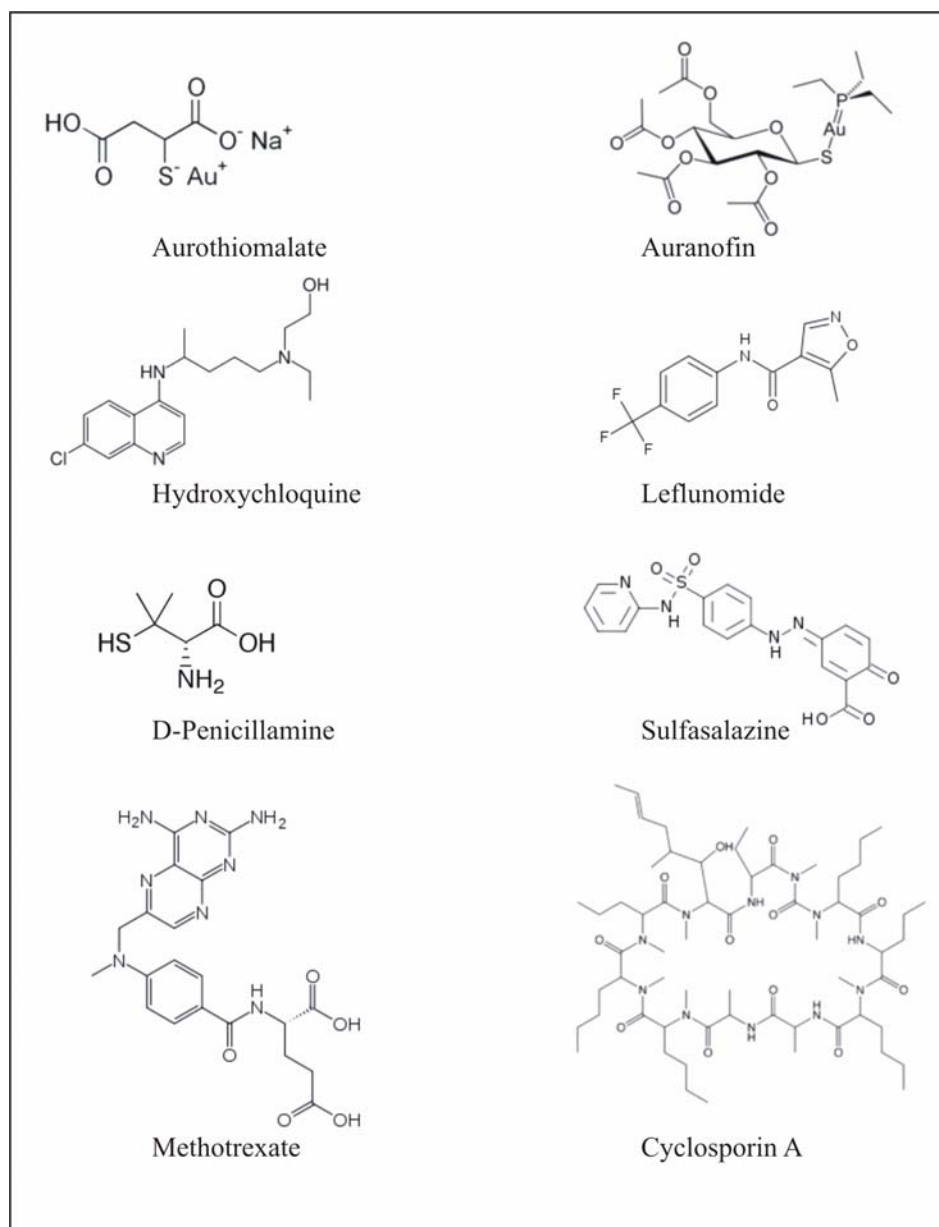

Figure 8. *Chemical structures of some DMARDs*

Gold sodium chloride was claimed to be effective in the treatment of tuberculosis and syphilis in Chrestin's 1811 publication. Robert Koch's finding in 1890 that gold cyanide was bactericidal *in vitro* to tubercle bacilli was a continuation of that approach (Koch 1890, Kean and Kean 2008). After that finding, gold complexes were used in the treatment of human and bovine tuberculosis. In the 1930's there was belief that RA and tuberculosis were related and a French physician, Jacques Forestier, began to treat his RA patients with gold. Forestier succeeded: gold complexes were found to be efficient and beneficial in the treatment of RA (Forestier 1934, Slot and Deville 1934, Forestier 1935)

According to the ACR guidelines for the management of rheumatoid arthritis, DMARD therapy should be started as soon as possible after RA diagnosis to prevent or slow further joint damage (Saag et al. 2008). With single DMARD therapy, remission is achieved in too few patients, and therefore the current practice is moving towards combination therapy (Pincus et al. 1999, Korpela et al. 2004). In the literature of DMARD combination therapy, methotrexate has been considered as the foundation on which sulfasalazine, hydroxychloroquine or biological agents are added. According to ACR guidelines for the management of RA, gold salts belong to a second line of drugs used in the treatment of RA (Saag et al. 2008). In the Finnish Current Care guidelines for RA, aurothiomalate is regarded as a useful drug also in the initiation of medical treatment for RA (Working group set up by the Finnish Rheumatological Society 2003).

Antirheumatic biologic agents are protein modulators that target specific proinflammatory cytokines (TNF-α, IL-1, IL-6) or inflammatory cell surface proteins. TNF-α blockers are the most widely used biologicals in the treatment of RA, and they act more quickly than traditional DMARDs and have been shown to slow the rate of radiographic progression. (Bathon et al. 2000, Lipsky et al. 2000) Interestingly, Rau compared four clinical trials with aurothiomalate (two open, one placebo-controlled, and one comparison with methotrexate) and five trials with biologic agents [(three placebo-controlled (anakinra, an IL-1

receptor antagonist; and two adalimumab, TNF-α blocker trials), one dose escalation study (infliximab, a TNF-α blocker trial), and one comparison with methotrexate (etanercept, a TNF-α blocker trial)] and concluded that aurothiomalate did not perform significantly worse in clinical trials than the biological agents (Rau 2005).

4.2 Aurothiomalate in non-clinical studies

Aurothiomalate has been shown to suppress lymphocyte transformation *in vitro* (Davis et al. 1979), and to inactivate the first component of complement (C_1) in serum, synovial fluid, and in functionally pure C_1 (Schultz et al. 1974). In rabbit liver lysosomes and in human synovial fluid aurothiomalate has been reported to inhibit secretion of hydrolytic enzymes, like acid phosphatase, β-glucuronidase and cathepsin (Ennis et al. 1968). Kean and co-workers have shown that aurothiomalate can modulate the interaction of a serine esterase enzyme, thrombin, in human platelets, human platelet-rich plasma and human platelet-poor plasma (Kean et al. 1984). This observation was confirmed *in vivo* in a rabbit model (Kean et al. 1991).

Aurothiomalate has been described to have an effect on various immunological responses. Aurothiomalate treatment was shown to significantly decrease the concentrations of circulating immunoglobulins (IgM and IgG) and rheumatoid factor (Gottlieb et al. 1975, Lorber et al. 1978) and it was also reported to affect cell mediated immune responses *in vitro* (Rosenberg and Lipsky 1979). De Wall and colleagues studied the effect of aurothiomalate on class II major histocompatibility complex (MHC) protein-peptide interactions and found out that aurothiomalate was capable of stripping peptides from MHC proteins and thus blocking the ability of antigen-presenting cells to activate T cells (De Wall et al. 2006). Wood and colleagues suggested that aurothiomalate's effect was due to the thiol groups in its structure. They proposed that reactive aldehydes may be common mediators of cell death in RA joint destruction and that thiol groups act to reduce this aldehyde load (Wood et al. 2008).

Aurothiomalate has been shown to inhibit PGE_2 production in peritoneal macrophages (Yamashita et al. 2003), rat astrocytes (Pistritto et al. 1999), human peripheral blood mononuclear cells (Ohta et al. 1986) and fibroblast-like synoviocytes (Stuhlmeier 2007). Stuhlmeier showed that aurothiomalate could block the release of PGE_2 and prevent the activation of NFκB leading to the inhibition of IL-1β-induced hyaluronan accumulation (Stuhlmeier 2007). Aurothiomalate did not seem to inhibit PGE_2 production in human polymorphonuclear leucocytes (Parente et al. 1986, Moilanen et al. 1988).

In addition to PGE_2 production, aurothiomalate seems to inhibit the synthesis of some other pro-inflammatory factors. It has been reported to suppress IL-8 production in endothelial cells, peripheral blood mononuclear cells (Seitz et al. 1992) and in rheumatoid synoviocytes (Loetscher et al. 1994) as well as to inhibit IL-12 production and IL-2 receptor expression in T cells (Sfikakis et al. 1993). Seitz et al. have described inhibition of IL-1β production and caspase-1 activity in THP-1 monocytes (Seitz et al. 2003). Aurothiomalate has also been shown to decrease serum IL-6 levels in RA patients (Lacki et al. 1995). In chondrocytes and in human OA cartilage aurothiomalate has been shown to inhibit iNOS expression and NO production (Vuolteenaho et al. 2005). Yanni et al. reported that administration of aurothiomalate resulted in reduced accumulation of monocytes and macrophages in RA synovial membranes and evoked a significant inhibition of TNF-α, IL-1β and IL-6 expression in these cells (Yanni et al. 1994). Gold has been shown to accumulate in endothelial cells and macrophages, and this may explain its direct toxicity to some organs (Nickels et al. 1983). Interestingly, Palosuo and co-workers showed that aurothiomalate therapy may facilitate the release of nephritis-associated ubiquitous tissue antigen into the circulation and that may lead to the manifestation of autoimmunity (Palosuo et al. 1978). Despite these findings, virtually nothing is known about the molecular effects of aurothiomalate on chondrocytes although it has been shown to retard cartilage degradation (Luukkainen et al. 1977a, Sander et al. 1999, Lehman et al. 2005).

4.3 Aurothiomalate in clinical studies

In the first clinical trials of gold compounds in the treatment of RA in 1929, Forestier treated 16 patients with gold thiopropanol sodium sulphonate given once a week by intramuscular injection. Five patients had an excellent response, five patients were much improved and two had a minimal response. For four other patients, the data about the clinical response is lacking. In 1930 Forestier continued with 33 RA patients and confirmed the results of the first study. (Kean et al. 1985) In 1934, Forestier presented the results of 50 RA patients treated with gold compounds, recording a 70 to 80 % success rate. He claimed that 50 % of patients treated early in the disease state were permanently recovered compared to only 25 % of patients who had had RA for two or more years. (Forestier 1934) In 1935, Forestier published results of 550 RA patients treated with gold compounds and confirmed earlier results (Forestier 1935). After that, uncontrolled studies of treatment of RA with gold compounds showed that 50-80 % of patients treated with gold compounds seemed to benefit from the treatment; meanwhile the adverse effects that occurred could be serious or even fatal (Snyder et al. 1939, Rawls et al. 1944).

In 1945, Fraser and colleagues published a double-blind controlled trial of aurothiomalate (this was the first, double-blind controlled trial of any anti-rheumatic drug) that confirmed Forestier's original findings and showed an efficacy rate of 82 % in aurothiomalate-treated patients compared to 45 % in the control patients (receiving inactive control compound) (Fraser 1945). In 1957 the Empire Rheumatism Council executed a multi-centre trial in 24 centers through the United Kingdom. In this study of 99 aurothiomalate-treated patients (receiving 50 mg aurothiomalate weekly for 20 weeks) and one hundred control patients (receiving 0.5 µg aurothiomalate weekly for the same time) it was clearly demonstrated that in most patients receiving the higher dose of aurothiomalate there was a progressive improvement in multiple variables, like the number of inflamed joints, grip strength and the erythrocyte sedimentation rate. (The Research sub-committee of the Empire Rheumatism Council 1960, The Research sub-committee of the Empire Rheumatism Council 1961) In 1973, the American Rheumatism Association confirmed these results in another

double-blind multi-centre trial with 68 patients with RA (36 of them receiving 50 mg aurothiomalate weekly for six months; 32 of them receiving sterile water) (Cooperating clinics committee of the American Rheumatism Association 1973).

Sigler and co-workers reported the results of a 2 year double-blind study of 13 RA patients receiving aurothiomalate compared to 14 RA patients receiving placebo. A significant improvement was reported in the aurothiomalate group. One notable facet of this study is the radiological evidence of arrest of bone and cartilage destruction and that the mean progression rate was significantly slowed in the aurothiomalate-treated group. (Sigler et al. 1974) The favourable effects of aurothiomalate on bone and cartilage erosions were confirmed in a much larger but uncontrolled study in 1977 (Luukkainen et al. 1977a). Luukkainen and colleagues reported also that the effects of aurothiomalate after 5 to 6 years were better in patients whose total aurothiomalate dosage was high (nearly 2 g) than in patients who had received a lower total dose since the treatment was discontinued because of adverse effects. They reported also that the earlier the treatment was initiated, the better was the result. (Luukkainen et al. 1977b)

Four trials and 415 patients were included in a Cochrane review about injectable gold for rheumatoid arthritis. In these studies, aurothiomalate was found to be significantly beneficial when compared to placebo. The number of swollen joints, erythrocyte sedimentation rate, and physicians' and patients' global assessments were all better in aurothiomalate treated subjects than in the placebo group. (Clark et al. 2000) Recently Lehman and colleagues published a 48-week, randomized, double-blind, placebo-controlled multi-centre study of combination methotrexate and aurothiomalate, and showed that in patients with a suboptimal response to methotrexate, addition of weekly aurothiomalate caused a significant clinical improvement (Lehman et al. 2005).

Based on the clinical studies presented above, aurothiomalate has been shown to effectively control RA symptoms and disease activity, and it has proved to be both anti-inflammatory and anti-erosive. Interestingly, when compared with biologic agents, aurothiomalate did not perform significantly worse in clinical

trials (Rau 2005). Although its efficacy is not in question, its clinical use has declined because of adverse effects and because of the recent rise in the use of biological DMARDs. However, biological therapies for RA are expensive and of little benefit in about 30% of cases, so that the pressing need for small molecule RA therapies remains. Some investigators have argued that the clinical value of gold compounds is due for reappraisal (Whitehouse 2008). At the same time, a deeper understanding of the therapeutic actions of aurothiomalate may lead to the identification of targets for new drugs for inflammatory joint diseases. Despite the long clinical history in the treatment of RA, a detailed knowledge of the molecular mechanism of action of aurothiomalate is still lacking.

Aims of the study

Enzyme COX-2 is highly expressed in RA and OA synovia and cartilage and produces high amounts of proinflammatory prostaglandins in the joint. MAP kinases are important signaling pathways mediating inflammatory signals to intracellular target proteins. Inhibitors of p38 and JNK MAPKs are under development for use in the treatment of arthritis and they have shown efficacy in experimentally induced arthritis and joint pain.

The aim of the present study was to investigate the role of the three MAP kinase pathways in the regulation of COX-2 expression and PGE_2 production in chondrocyte and macrophage cell lines and in intact cartilage as potential targets for drug development. Another major aim was to investigate the effects of DMARDs on COX-2 expression and further to study the mechanism of action of aurothiomalate in more detail.

The detailed aims were:

1. to investigate the effects of MAPK inhibitors on COX-2 expression and PGE_2 production in chondrocytes and macrophages (I, II)

2. to investigate the effects of DMARDs on COX-2 expression and PGE_2 production in chondrocytes and in intact human cartilage. And further, to investigate the effect of aurothiomalate on MMP-3 and IL-6 expression (III, IV)

3. to test the hypothesis that aurothiomalate down-regulates COX-2, MMP-3 and IL-6 expression by increasing MKP-1 expression and decreasing p38 MAPK phosphorylation in chondrocytes and cartilage by using MKP-1 siRNA and MKP-1$^{-/-}$ mice (IV)

Materials and methods

1 Reagents

1.1 Antibodies

The reagents used in this study were obtained as follows: goat polyclonal mouse and human anti-COX-2, rabbit polyclonal anti-c-Jun, anti-JNK1, anti-MKP-1, and anti-actin, donkey anti-goat IgG and goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-phospho-JNK (Thr183/Tyr185), anti-phospho-Erk1/2 (Thr202/Tyr204), anti-Erk1/2, anti-phospho-p38 (Thr180/Tyr182), anti-p38 and anti-phospho-c-Jun (Ser63) antibodies (Cell Signaling Technology Inc., Beverly, MA, USA).

1.2 Chemicals

PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one, Erk1/2 inhibitor), SB203580 (4-(4fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazol, p38 inhibitor), SB202474 (4-ethyl-2-(4-methoxyphenyl)-5-(4-hydroxyphenyl)-5-(4pyridyl)-imidazole, inactive control compound for p38 inhibitor), SP600125 (anthra(1,9-cd)pyrazol-6(2H)-one, JNK inhibitor), N1-methyl-substituted pyrazolanthrone (N1-methyl-1,9-pyrazolanthrone, inactive control compound for JNK inhibitor) and cyclosporin A were obtained from Calbiochem (San Diego, CA, USA). Dexamethasone and methotrexate were kindly provided by Orion Corporation. (Espoo, Finland). Human recombinant IL-1β was purchased from R&D Systems Inc. (Minneapolis, MA, USA). All other reagents were obtained from Sigma (Sigma Chemical Co., St Louis, MO, USA). Pharmacological compounds used in this study are presented in Table 3.

Table 3. *Pharmacological compounds used in the present study.*

Compound	
A771726	Active metabolite of leflunomide
Actinomycin D	RNA polymerase II inhibitor
Alsterpaullone	CDK2 inhibitor
Aurothiomalate	DMARD
Cyclophosphamide	JNK activator and an inhibitor of protein synthesis
Cyclosporin A	DMARD
Hydroxychloroquine	DMARD
Indirubin-3'-monoxime	CDK2 inhibitor
Leflunomide	DMARD
Methotrxate	DMARD
N1-methyl substituted pyrazolanthrone	Negative control compound for SP600125
PD98059	MKK 1/2 inhibitor, inhibits ERK1/2 activation
D-Penicillamine	DMARD
SB202474	Negative control compound for SB203580
SB203580	p38 inhibitor
Sodium orthovanadate	Tyrosine phosphatase inhibitor
SP600125	JNK inhibitor
Sulfasalazine	DMARD

2 Tissue and cell cultures

J774A.1 murine macrophages (American Type Culture Collection, Rockville, MD, USA), immortalized human T/C28a2 chondrocytes (Goldring et al. 1994) (kindly provided by Prof. Mary B. Goldring, Harvard Institutes of Medicine, Boston, MA, USA) and immortalized murine H4 chondrocytes (van Beuningen et al. 2002) (kindly provided by Dr Hank van Beuningen and Prof. Peter M. van der Kraan, University Medical Center Nijmegen, Nijmegen, The Netherlands) were grown at 37°C in 5% CO_2 atmosphere, in Dulbecco's modified Eagle's medium with glutamax-I (Cambrex Bioproducts Europe, Verviers, Belgium) (J774A.1 cells) and in Dulbecco's modified Eagle's medium (Cambrex Bioproducts Europe, Verviers, Belgium) and Ham's F-12 medium (Gibco,

Paisley, Scotland) (1:1, v/v) (T/C28a2 and H4 cells), containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (all from Gibco, Paisley, Scotland). Cells were seeded on 24-well plates for PGE_2 measurements and on 24-well or six-well plates for Western blot and RT-PCR. Cell monolayers were grown for 48 h (H4) or 72 h (J774A.1 and T/C28a2) to confluence before the experiments were started and the compounds of interest were added in fresh culture medium.

Cartilage tissue was obtained from the leftover pieces of total knee replacement surgery from patients with OA or RA. The study was approved by the ethics committee of Tampere University Hospital and the patients gave their written approval. Full thickness pieces of articular cartilage from femoral condyles, tibial plateaus and patellar surfaces were removed aseptically from subchondral bone with a scalpel and cut into small pieces. The cartilage samples were incubated at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (all from Gibco, Paisley, Scotland, U.K.). The experimental procedure was done within a few hours after the operation. Cartilage samples were incubated on six-well plates for PGE_2 and Western blot measurements for 24-48 h.

Inbred C57BL/6 MKP-1$^{-/-}$ and wild type mice were a kind gift from Dr. Andrew Clark (Kennedy Institute of Rheumatology, London) and were originally generated by the R. Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute (Dorfman et al. 1996). The study was approved by the Animal Care and Use Committee of the University of Tampere and the respective provincial committee for animal experiments. Female mice aged 10-12 weeks were used in the study. The mice were anesthesized by intraperitoneal injection of 0.05 mg/100 g body weight of medetomine (Domitor® 1 mg/ml, Orion Oyj, Espoo, Finland) and 7.5 mg/100 g body weight of ketamine (Ketalar® 10 mg/ml, Pfizer Oy Animal Health, Helsinki, Finland). Subsequently, the mice were euthanized by decapitation. Cartilage tissue was taken from the knees of mice hind legs. Full thickness pieces of articular cartilage from femoral condyles,

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tibial plateaus and patellar surfaces were removed aseptically from subchondral bone with a scalpel. The cartilage samples were incubated at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (all from Gibco, Paisley, Scotland). Cartilage samples were incubated on 24-well plates for PGE_2, cytokine and Western blot measurements and incubated for 48 h.

3 Western blotting

At the indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and solubilized in cold lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 μM n-octyl-β-D-glucopyranoside). Cartilage samples were incubated for 24-48 h, after incubation deep frozen in liquid nitrogen and then crushed into powder. Powder was added to cold lysis buffer and samples for Western blot analysis made as described below.

Following incubation for 20 min on ice, lysates were centrifuged, and supernatants were mixed in a ratio of 1:4 with SDS loading buffer (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 -20°C until analyzed. The protein concentration of the samples was measured by the Coomassie blue method (Bradford 1976).

The samples were boiled for 10 min prior to Western blot analysis, equal aliquots of protein (20 μg) were loaded on a 10 % (COX-2) or 12 % (JNK, p38, Erk1/2, c-Jun, pJNK, pp38, pErk1/2, MKP-1) SDS-polyacrylamide electrophoresis gel and electrophoresed for 1 h at 120 V in a buffer containing 95 mM Tris-HCl, 960 mM glycine, and 0,5% SDS. After electrophoresis, the proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose

membrane (Amersham, Buckinghamshire, U.K.) with semidry blotter at 2.5 mA/cm2 for 60 min. After transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat milk for 1 h at room temperature and incubated overnight at 4º C with primary antibody in TBS/T containing 5% nonfat milk. Thereafter the membrane was washed four times with TBS/T for 5 min, incubated with secondary antibody coupled to horseradish peroxidase in the blocking solution for 0.5 h at room temperature, and washed four times with TBS/T for 5 min. Bound antibody was detected using SuperSignal West Pico chemiluminescent substrate (Pierce, Cheshire, U.K.) and FluorChem 8800 imaging system (Alpha Innotech Corp., San Leandro, CA, USA). The quantitation of the chemiluminescent signal was carried out with the use of FluorChem software version 3.1.

4 RNA extraction

At the indicated time points, cell monolayers were rapidly washed with ice-cold PBS, and cells were homogenized and purified using QIAshredderTM (QIAGEN Inc., Valencia, CA, USA). RNA extraction was carried out with the use of RNeasy® kit for isolation of total RNA (QIAGEN Inc.).

5 Real-time PCR

Total RNA (25 or 100 ng) was reverse-transcribed to cDNA using TaqMan® Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). Parametres for the reverse-transcribe (RT) reaction were: incubation at 25°C for 10 min, RT at 48°C for 30 min, and RT inactivation at 95°C for 5 min. cDNA obtained from the RT reaction (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 and concentrations were optimized according to the manufacturer's guidelines in TaqMan Universal PCR

Master Mix Protocol part number 4304449 revision C. The primers and probes used in this study are listed in Table 4. Human β-actin was obtained from TaqMan Human β-actin Reagents kit (Applied Biosystems), containing VIC as 5'-reporter dye and TAMRA as 3'-quencher. PCR reaction parameters were: incubation at 50ºC for 2 min, incubation at 95ºC for 10 min, and thereafter 40 cycles of denaturation at 95ºC for 15 s and annealing and extension at 60ºC for 1 min. Each sample was determined in duplicate.

Table 4. *Primer and probe sequences used in quantitative real-time PCR..*

5'-CAACTCTATATTGCTGGAACATGGA-3'	forward human COX-2 primer (300 nM)
5'-TGGAAGCCTGTGATACTTTCTGTACT-3'	reverse human COX-2 primer (300 nM)
5'-TCCTACCACCAGCAACCCTGCCA-3'	human COX-2 probe (150 nM)
5'-GCCAGGGCTGAACTTCGAA-3'	forward mouse COX-2 primer (300 nM)
5'-CAATGGGCTGGAAGACATATCAA-3'	reverse mouse COX-2 primer (300 nM)
5'-CTCACGAGGCCACTGATACCTATTGCATTG-3'	mouse COX-2 probe (150 nM)
5'-GCATGGCCTTCCGTGTTC-3'	forward mouse GAPDH primer (300 nM)
5'-GATGTCATCATACTTGGCAGGTTT-3'	reverse mouse GAPDH primer (300 nM)
5'-TCGTGGATCTGACGTGCCGCC-3'	mouse GAPDH probe (150 nM)
5'-TGTCCCCGGCAATGCT-3'	forward mouse HuR primer (300 nM)
5'-TCACGAATCACTTTCACATTGGT-3'	reverse mouse HuR primer (300 nM)
5'-CCTCATCGGCGTCTTGCCCAA-3'	mouse HuR probe (150 nM)
5'-CAAGGATGCTGGAGGGAGAGT-3'	forward mouse MKP-1 primer (300 nM)
5'-TGAGGTAAGCAAGGCAGATGGT-3'	reverse mouse MKP-1 primer (300 nM)
5'-TTTGTTCATTGCCAGGCCGGCAT-3'	mouse MKP-1 probe (150 nM)
5'-CTCAGAAAGCGGGCGTTGT-3'	forward mouse TTP primer (300 nM)
5'-GATTGGCTTGGCGAAGTTCA-3'	reverse mouse TTP primer (300 nM)
5'-CCAAGTGCCAGTTTGCTCACGGC-3'	mouse TTP probe (200 nM)

A standard curve method was used to determine the relative mRNA levels as described in the Applied Biosystems User Bulletin number 2: A standard curve for each gene was created using RNA isolated from IL-1β-stimulated human T/C28a2 chondrocytes or LPS-stimulated J774 macrophages. Isolated RNA was reverse-transcribed as described and dilution series were made from the obtained cDNA, ranging from to 1 pg to 10 ng and were subjected to real-time PCR. 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.

6 siRNA transfection to knockdown MKP-1

H4 chondrocytes were transfected with MKP-1-specific siRNA, ON-TARGET SMART pool consisting of four siRNAs or negative control using Dharmafect I. To down-regulate MKP-1, the cells were seeded at 2.5 x 10^5 cells per well in 24-well plates. Cells were incubated 24 h and then transfected with siRNA oligos targeted to murine MKP-1 or negative control. After 24 h, culture medium was changed to serum free, and after another 24 h incubations, the experiments were started and IL-1β and the tested compounds were added in fresh culture medium. Transfection efficacy was monitored with green fluorescent siRNA oligos (siGLO green indicator). Transfection efficacy was found to be 70-80 %. All oligos and transfection reagents were from Dharmacon Research (Dharmacon Research, Lafayette, CO, USA).

7 Determination of cell viability

Cell viability was tested by using colorimetric Cell Proliferation Kit II (Roche Diagnostics GmbH, Mannheim, Germany) that measures the cells' ability to metabolise a tetrazolium salt XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate) to formazan

by mitochondrial dehydrogenase activity that only occur in viable cells. Cells were seeded on 96-well plates and incubated for 20 h at 37°C with the tested compounds. XTT (0.3 mg/ml) 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 in culture medium was then measured spectrophotometrically. Triton-X treated cells were used as a positive control.

8 PGE_2 measurements

PGE_2 concentrations were determined by radioimmunoassay using reagents from the Institute of Isotopes (Budapest, Hungary). Cartilage tissue pieces were weighed and the results were expressed as ng of PGE_2 / g of cartilage tissue.

9 Enzyme-linked immunosorbent assay (ELISA)

The concentration of human IL-6 (PeliPair® ELISA, Sanquin, Amsterdam, the Netherlands), murine IL-6, human MMP-3 (DuoSet® ELISA, R&D Systems Europe Ltd, Abindgon, U.K.) and murine MMP-3 (Quantikine® ELISA, R&D Systems Europe Ltd, Abindgon, U.K.) were determined by ELISA according to the manufacturer's instructions.
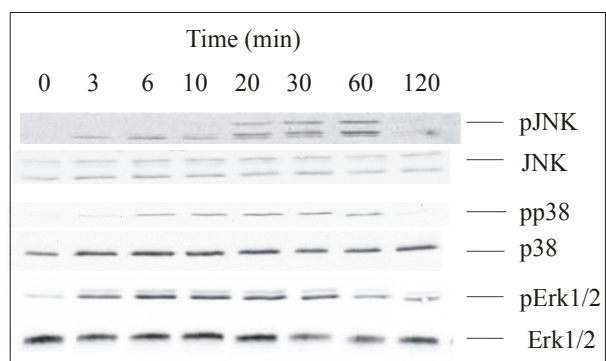
10 Statistics

Results of cell and tissue culture experiments are expressed as the mean ± standard error of mean (S.E.M). When appropriate, statistical significance was calculated by analyses of variance followed by Dunnett multiple comparisons test. Differences were considered significant when $P < 0.05$.

Summary of the results

1. IL-1β and LPS activate MAPKs and induce COX-2 expression and PGE$_2$ production in chondrocytes, cartilage and macrophages (I-IV)

Resting cells (human T/C28a2 chondrocytes, murine J774 macrophages and murine H4 chondrocytes) did not contain detectable levels of phophorylated MAP kinases indicating low levels of active MAPKs. Stimulation with proinflammatory cytokine IL-1β (I, IV) or bacterial endotoxin LPS (II) rapidly enhanced phosphorylation of all the three studied MAPKs (p38, JNK and Erk1/2). In T/C28a2 chondrocytes p38 activation peaked at 20 min and in H4 chondrocytes at 30 min after IL-1β. JNK peaked at 1 h after IL-1β in T/C28a2 chondrocytes, at 30 min after IL-1β in H4 chondrocytes and at 30 min after LPS in J774 macrophages. Erk1/2 peaked at 20 min after IL-1β in T/C28a2 chondrocytes. The levels of phosphorylated MAPKs also reduced rapidly after the peak, and in most cases had returned close to the pre-stimulation levels within 2 h after the stimulus. (Figure 9)

Figure 9. *The effect of IL-1β on JNK, p38, and Erk1/2 MAPK phosphorylation in human T/C28a2 chondrocytes. The chondrocytes were stimulated with IL-1β (100 pg/ml). Incubations were terminated at the indicated time points. Two parallel immunoblots were run from same cell lysates using antibodies against phosphorylated JNK (pJNK), p38 (pp38), and Erk1/2 (pErk1/2) and against total JNK, p38, and Erk1/2.* (Reprinted with permission from: Nieminen et al. 2005, Mediators Inflamm 2005:249-255 © Hindawi Publishing Corporation)

In unstimulated cells and cartilage, COX-2 mRNA and protein expression and PGE$_2$ production remained at low levels. IL-1β (in chondrocytes and cartilage) and LPS (in macrophages) stimulated COX-2 expression and PGE$_2$ production in a dose- and time-dependent manner. The peak in COX-2 mRNA levels was reached at 4 h (T/C28a2 and H4 chondrocytes) and at 6 h (J774 macrophages) after stimulus. In H4 chondrocytes, the COX-2 mRNA levels rapidly decreased after 4 h and reached basal level at 8 h whereas in T/C28a2 chondrocytes (Figure 10A) and in J774 macrophages COX-2 mRNA levels slowly decreased to about 50 % of the peak level and remained elevated during the 48 h follow-up. The COX-2 protein expression was at its maximum at 8 h after IL-1β in T/C28a2 chondrocytes (Figure 10B) and at 24 h after IL-1β or LPS in H4 chondrocytes and in J774 macrophages, respectively. PGE$_2$ production reached its maximum in all cells used at 24 h after addition of stimulus (Figure 10C). In cartilage cultures, COX-2 expression and PGE$_2$ levels in the culture medium reached maximum at 48 h after IL-1β. Treatment with a COX inhibitor (ibuprofen 10 μM) completely inhibited IL-1β or LPS –induced PGE$_2$ production.
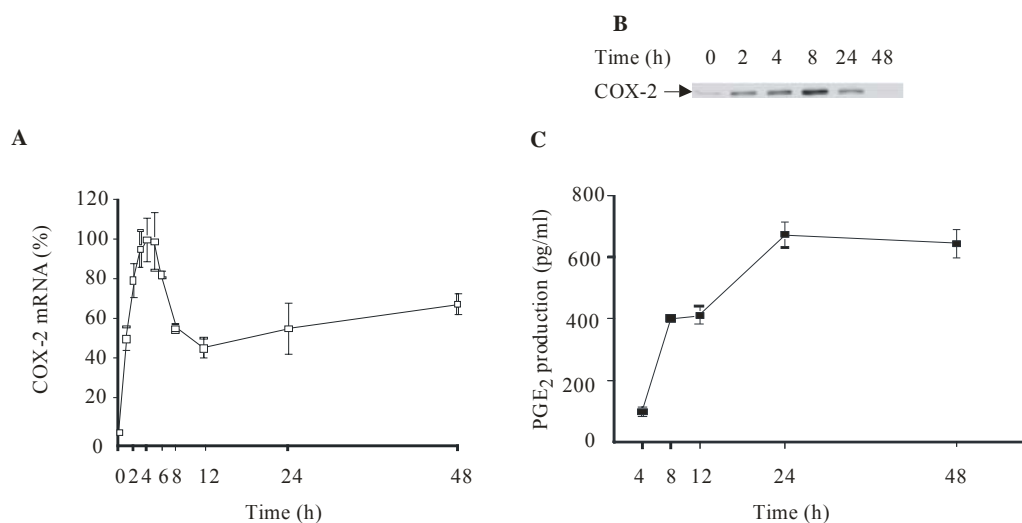

Figure 10. The effect of IL-1β on COX-2 mRNA and protein expression and PGE$_2$ production in human T/C28a2 chondrocytes. (A) Cells were stimulated with IL-1β (100 pg/ml). Incubations were terminated at time points indicated and extracted total RNA was subjected to real time RT-PCR. COX-2 mRNA levels were normalized against GAPDH. Values are mean ± SEM, n=3. (B) Cells were stimulated with IL-1β (100 pg/ml). Incubations were terminated at indicated time points and COX-2 detected by Western blot. (C) Cells were stimulated with IL-1β (100 pg/ml). Culture medium was collected at the indicated time points and PGE$_2$ concentrations were measured by RIA. Values are mean ± SEM, n=6. (Reprinted with permission from: Nieminen et al. 2005, Mediators Inflamm 2005:249-255 © Hindawi Publishing Corporation, modified)

2. MAPK pathways regulate COX-2 expression and PGE$_2$ production (I, II, IV)

SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) and PD98059 (inhibitor of ERK1/2 activation) were used to inhibit the three better characterized MAP kinase pathways.

First we investigated the effects of MAPK inhibitors on COX-2 expression and subsequent PGE$_2$ production in human T/C28a2 chondrocytes and found out that all used inhibitors (SP600125, SB203580 and PD98059) inhibited COX-2 expression and PGE$_2$ production in a dose-dependent manner (Figure 11).
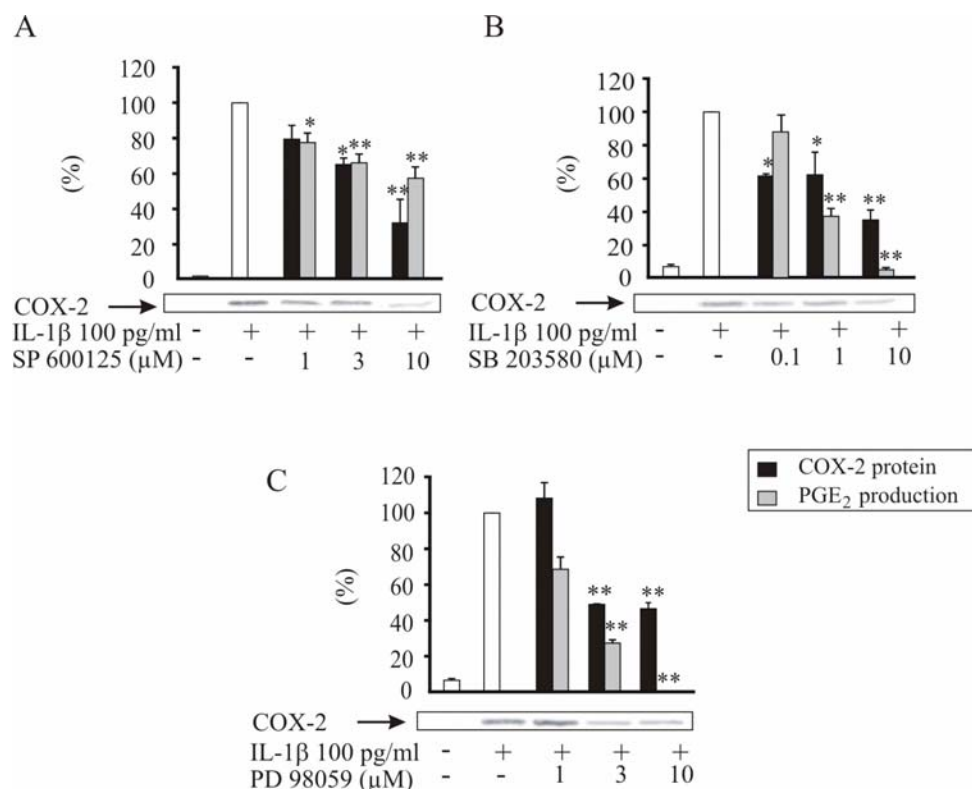

*Figure 11. The effects of MAP kinase inhibitors SP600125, SB203580 and PD98059 on COX-2 protein expression and PGE$_2$ production in IL-1β stimulated human T/C28a2 chondrocytes. Human chondrocytes were incubated with IL-1β (100 pg/ml) and increasing concentrations of SP600125 (JNK inhibitor; A), SB203580 (p38 inhibitor; B) and PD98059 (Erk1/2 inhibitor; C). After 24 h, incubations were terminated and COX-2 protein and PGE$_2$ concentration were measured by Western blot and radioimmunoassay, respectively. Values are mean ± SEM, n=4, ** indicates P<0.01, * indicates P<0.05 as compared with cells treated with IL-1β alone. (Reprinted with permission from: Nieminen et al. 2005, Mediators Inflamm 2005:249-255 © Hindawi Publishing Corporation, modified)*

The effect of MAPK inhibitors on COX-2 mRNA was tested at two time points, 2 and 8 h after addition of IL-1β. In this experimental setting, the effect of JNK inhibitor differed from those of the p38 and ERK1/2 inhibitors. SB203580 and PD98059 inhibited COX-2 mRNA when measured 2 h after addition of IL-1β and the inhibitory effect was stronger at the 8 h time point whereas SP600125 inhibited COX-2 mRNA expression only at the 8 h time point (Figure 12).
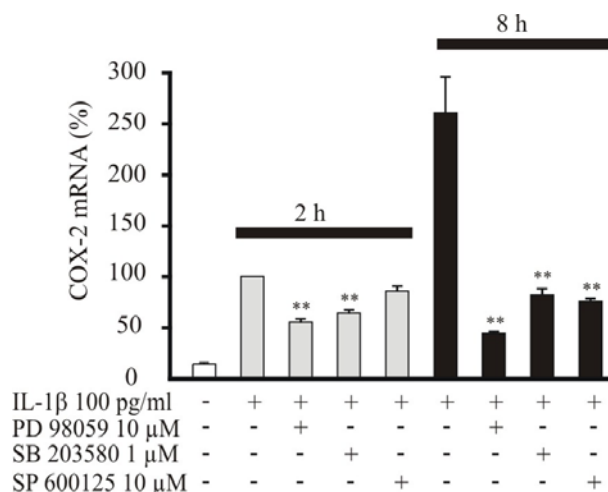

Figure 12. *The effects of SP600125, SB203580 and PD98059 on COX-2mRNA levels in IL-1β stimulated human T/C28a2 chondrocytes. Human chondrocytes were incubated with IL-1β (100 pg/ml) and with or without SP600125, SB203580 and PD98059. Incubations were terminated at the indicated time points, and the extracted total RNA was subjected to real-time RT-PCR. COX-2 mRNA levels were normalized against β-actin mRNA. Values are mean ± SEM, n=6, ** indicates P<0.01 as compared to cells treated with IL-1β only. (Reprinted with permission from: Nieminen et al. 2005, Mediators Inflamm 2005:249-255 © Hindawi Publishing Corporation)*

SP600125 inhibited LPS-induced COX-2 expression and PGE$_2$ production also in macrophages. In agreement with the finding in T/C28a2 chondrocytes, JNK inhibitor did not inhibit COX-2 mRNA expression at the early time point (3 h) whereas at the later time point (24 h) the inhibition was significant. These results suggested that SP600125 did not affect the early transcriptional events involved in COX-2 expression but may rather regulate COX-2 mRNA stability. This hypothesis was investigated in mRNA degradation assay with actinomycin D. Cells were treated with LPS or a combination of LPS and SP600125 for 6 h (the peak level of COX-2 mRNA in these cells), and thereafter an inhibitor of transcription, actinomycin D, was added to the culture medium. Total RNA was extracted before, and 2, 4, and 6 h after addition of actinomycin D. In the

absence of actinomycin D, COX-2 mRNA levels declined faster in cells treated with a combination of SP600125 and LPS than in cells treated with LPS alone. However, actinomycin D inhibited COX-2 mRNA degradation and SP600125 had no effect in the presence of actinomycin D. (Figure 13A) In murine H4 chondrocytes, the p38 inhibitor (SB203580) destabilized IL-1β –induced COX-2 mRNA as measured by the actinomycin D assay (Figure 13B).
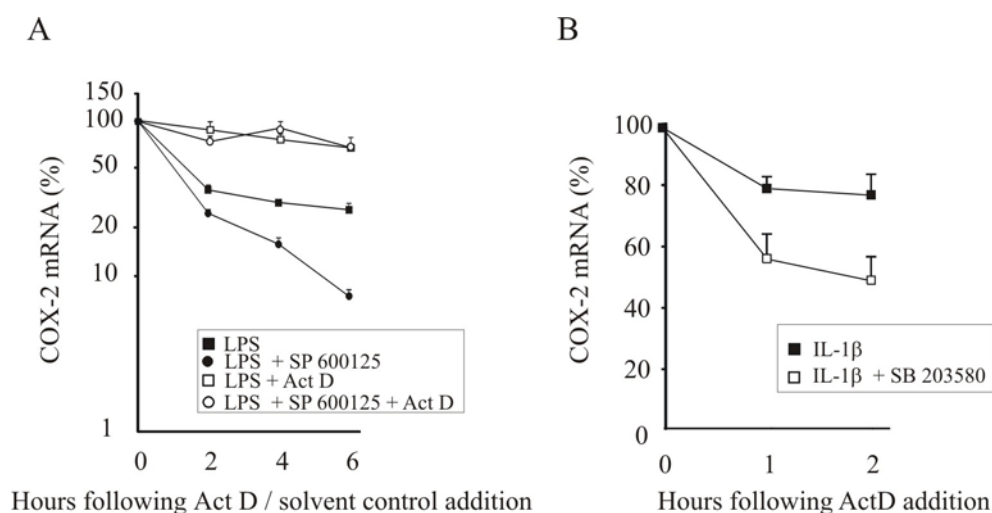

Figure 13. *The effects of SP600125 and SB203580 on COX-2mRNA decay. (A) J774 macrophages were incubated with LPS (10 ng/ml) or with a combination of LPS and SP600125 (10 μM). After 6 h, an inhibitor of transcription actinomycin D (1 μg/ml) or a solvent control was added into the cell culture. (B) H4 chondrocytes were incubated with IL-1β (100 pg/ml) or with a combination of IL-1β and SB230580 (1 μM). After 4 h actinomycin D (1 μg/ml) was added into the cell culture.*
Incubations were terminated at the indicated time points and the extracted total RNA was subjected to RT-PCR. COX-2 mRNA levels were normalized against GAPDH mRNA. Values are mean ± SEM, n=3. (Reprinted with permission from: Nieminen et al. 2006, Int Immunopharmacol 6:987-996 © Elsevier Ltd., modified)

3. Aurothiomalate inhibits COX-2 expression and PGE$_2$ production, and enhances degradation of COX-2 mRNA (III,IV)

The effects of six DMARDs (aurothiomalate, cyclosporin A, hydroxychloroquine, leflunomide, its metabolite A771726, methotrexate and sulfasalazine) were studied on COX-2 expression in H4 chondrocytes. Aurothiomalate inhibited COX-2 expression by 54 % whereas other tested DMARDs had a minor or no effect (Table 5). In the subsequent studies, aurothiomalate's effects and mechanisms of action were investigated in more detail.

Table 5. *Effects of disease modifying anti-rheumatic drugs on IL-1β -induced COX-2 expression in H4 chondrocytes.*

Compound	Concentration (μM)	COX-2 protein (% of IL-1β induced)
IL-1β		100
+ Aurothiomalate	10	46.1 ± 6.8 [**]
+ Methotrexate	10	76.8 ± 2.0
+ A771726	10	78.9 ± 9.3
+ Sulfasalazine	10	87.7 ± 10.7
+ Cyclosporin A	10	89.7 ± 17.6
+ Leflunomide	10	95.2 ± 1.4
+ Hydroxychloroquine	10	100.1 ± 11.6

Cells were incubated for 24 h with the tested DMARD (10 μM) and IL-1β (100 pg/ml). COX-2 protein was measured by Western blot. Results are expressed as mean ± SEM, n=3. [**] indicates P<0.01 as compared with cells treated with IL-1β alone. (Reprinted with permission from: Nieminen et al. 2008, Eur J Pharmacol 587:309-316 © Elsevier Ltd.)

Aurothiomalate reduced IL-1β -induced COX-2 protein expression and PGE$_2$ production in a concentration dependent manner in H4 chondrocytes (Figure 14A,B). Gold was found to be the effective component in aurothiomalate's

ability to inhibit COX-2 expression and PGE$_2$ production; thiomalic acid itself did not inhibit COX-2 expression whereas the inhibitory effect of the combination of gold chloride and thiomalic acid was similar to that of gold chloride alone which was close to that of aurothiomalate (Figure 14C,D).
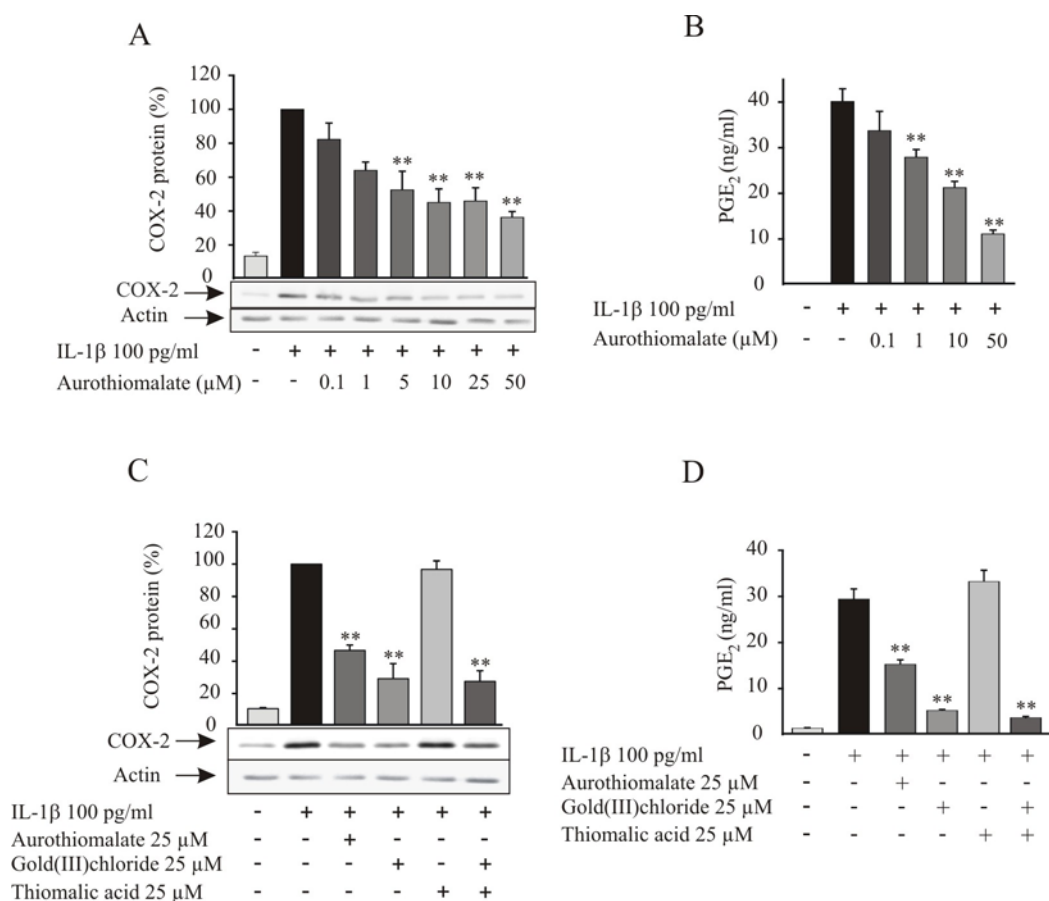

Figure 14. *The effect of aurothiomalate on IL-1β –induced COX-2 protein expression and PGE$_2$ production in H4 chondrocytes. (A,B) Chondrocytes were incubated with IL-1β (100 pg/ml) and with increasing concentrations of aurothiomalate. After 24 h, incubations were terminated and COX-2 protein was measured by Western blot (A) and PGE$_2$ concentrations in the culture medium were measured by RIA (B). (C,D) Chondrocytes were stimulated with IL-1β (100 pg/ml) and treated with aurothiomalate (25 μM), gold chloride (25 μM), thiomalic acid (25 μM) or a combination of gold chloride (25 μM) and thiomalic acid (25 μM). After 24 h, incubations were terminated and COX-2 protein was measured by Western blot (C) and PGE$_2$ concentrations in the culture medium were measured by RIA (D). Values are mean ± SEM, n=3-4. ** indicates P<0.01 as compared with cells treated with IL-1β alone. (Reprinted with permission from: Nieminen et al. 2008, Eur J Pharmacol 587:309-316 © Elsevier Ltd.)*

With respect to the mRNA levels, aurothiomalate had no effect on COX-2 mRNA expression when measured 3 h after addition of IL-1β, but had a marked inhibitory effect when measured 6 h after IL-1β. This data suggested that

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aurothiomalate does not probably regulate COX-2 transcription, but instead may rather regulate COX-2 mRNA stability. The hypothesis was tested in the COX-2 mRNA degradation assay, in which COX-2 mRNA levels decreased faster in cells treated with IL-1β in combination with aurothiomalate than in cells treated with IL-1β alone in conditions where transcription was inhibited by actinomycin D. Interestingly, the results with aurothiomalate were in line with these obtained with the p38 MAPK inhibitor SB203580. COX-2 mRNA half-life was about 3 h in cells treated with IL-1β only, it was reduced to less than 1.5 h in cells treated with a combination of IL-1β and aurothiomalate and it was about 2 h in cells treated with a combination of IL-1β and SB203580. (Figure 15)
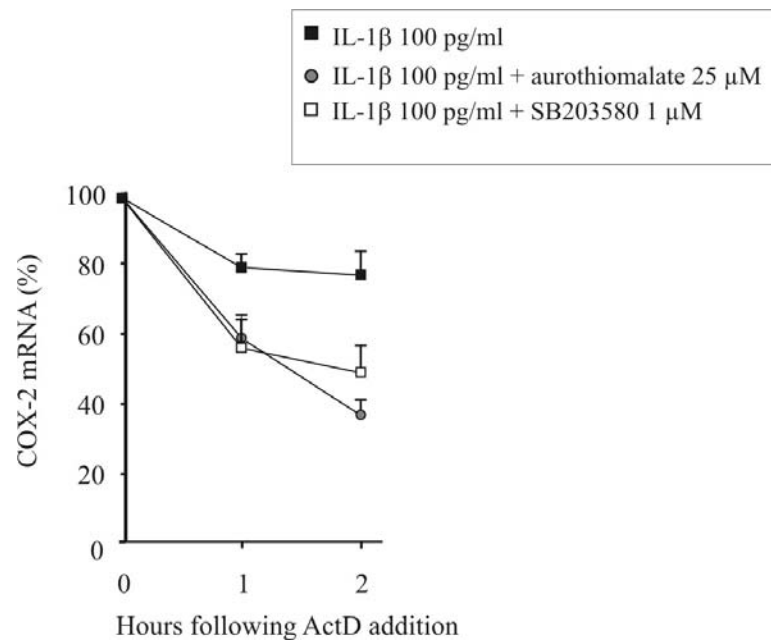

Figure 15. *The effect of aurothiomalate and SB203580 on COX-2 mRNA decay. H4 chondrocytes were incubated with IL-1β (100 pg/ml) or with a combination of IL-1β and aurothiomalate (25 μM) or a combination of IL-1β and SB203580 (1 μM). After 4 h actinomycin D (1 μg/ml) was added into the cell culture.*
Incubations were terminated at the indicated time points and the extracted total RNA was subjected to RT-PCR. COX-2 mRNA levels were normalized against GAPDH mRNA. Values are mean ± SEM, n=3.(Reprinted with permission from: Nieminen et al. 2008, Eur J Pharmacol 587:309-316 © Elsevier Ltd., modified)

4. Aurothiomalate enhances MKP-1 expression and reduces p38 MAPK phosphorylation (IV)

In HeLa cells, dexamethasone has been shown to destabilize COX-2 mRNA by decreasing p38 activity and increasing MKP-1 expression (Lasa et al. 2000, Lasa et al. 2001, Lasa et al. 2002). Aurothiomalate and the p38 inhibitor had similar effects on IL-1β -induced COX-2 expression and both had a destabilizing effect on COX-2 mRNA. On the basis of these results, we hypothetized that the effects of p38 and aurothiomalate may be somehow coupled. Interestingly, aurothiomalate reduced levels of phosphorylated p38 MAPK in IL-1β –treated cells (Figure 16A). Because that could be due to enhanced dephosphorylation of p38, we tested the effect of aurothiomalate on MKP-1 expression. The results showed that aurothiomalate enhanced MKP-1 expression by about 3-fold compared to cells treated with IL-1β alone (Figure 16B). Gold was found to be the effective component in aurothiomalate's ability to induce MKP-1 expression; thiomalic acid itself did not enhance MKP-1 expression whereas the effect of the combination of gold chloride and thiomalic acid was similar to that of gold chloride alone which was comparable to that of aurothiomalate (Figure 16C).
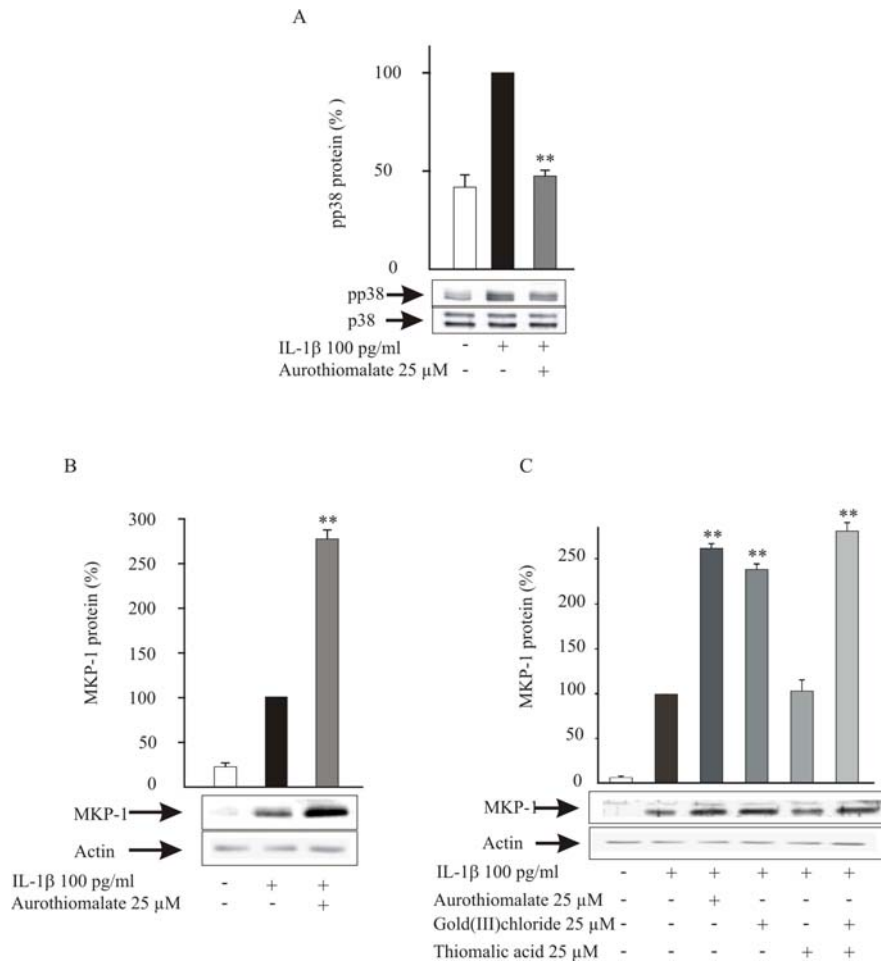
Figure 16. *The effect of aurothiomalate on p38 MAPK phosphorylation and MKP-1 expression.* (A) H4 chondrocytes were incubated with IL-1β (100 pg/ml) or with a combination of IL-1β (100 pg/ml) and aurothiomalate (25 µM). After 1 h, incubations were terminated and phosphorylated (pp38) and total p38 were measured by Western blot. (B) H4 chondrocytes were incubated with IL-1β (100 pg/ml) or with a combination of IL-1β (100 pg/ml) and aurothiomalate (25 µM). After 1.5 h, the incubations were terminated and MKP-1 protein was measured by Western blot. (C) Chondrocytes were stimulated with IL-1β (100 pg/ml) and treated with aurothiomalate (25 µM), gold chloride (25 µM), thiomalic acid (25 µM) or a combination of gold chloride (25 µM) and thiomalic acid (25 µM). After 1.5 h, incubations were terminated and MKP-1 protein was measured by Western blot.
Values are mean ± SEM, n=4. **indicates P<0.01 as compared to cells treated with IL-1β alone.

5. MKP-1 mediates the effects of aurothiomalate on COX-2, MMP-3 and IL-6 expression (IV)

Based on the results described above, we hypothesized that MKP-1 mediates the effects of aurothiomalate on COX-2 expression and PGE_2 production. The involvement of MKP-1 in the mechanism of action of aurothiomalate was first investigated by using sodium orthovanadate (an inhibitor of tyrosine phosphatase) to inhibit MKP-1 activity. Sodium orthovanadate (10 µM) itself slightly increased IL-1β -induced COX-2 expression. In the presence of sodium orthovanadate, aurothiomalate did not have any statistically significant effect on COX-2 expression in H4 chondrocytes whereas in the absence of sodium orthovanadate, aurothiomalate inhibited COX-2 expression by 55 % (Figure 17A).

To further investigate the role of MKP-1 on aurothiomalate's effect on COX-2 expression, the experiment was repeated in H4 chondrocytes which were transiently transfected with MKP-1 -specific siRNA to down-regulate MKP-1 expression. As a control for the MKP-1 siRNA, a sequence that was not complementary to any known mRNAs was transfected (negative control, siNEG). MKP-1 expression was clearly enhanced in response to IL-1β and IL-1β plus aurothiomalate in cells transfected with siNEG but to a much lesser extent if at all, in cells transfected with MKP-1 siRNA. Accordingly, aurothiomalate reduced p38 phosphorylation in siNEG –treated cells but not in MKP-1 siRNA– treated cells (Figure 17B).

In cells transfected with MKP-1 siRNA and treated with IL-1β and aurothiomalate, aurothiomalate's inhibitory effect on COX-2 expression was diminished from 50 % (p<0.01) in siNEG cells to 20 % (n.s.) in MKP-1 siRNA cells at the protein level and from 40 % (p<0.01) in siNEG cells to 10 % (n.s.) in MKP-1 siRNA cells at the mRNA level. In cells transfected with siNEG, the inhibitory effect of aurothiomalate was similar to that in non-transfected (WT) cells both at the protein and mRNA levels (Figure 17C,D).
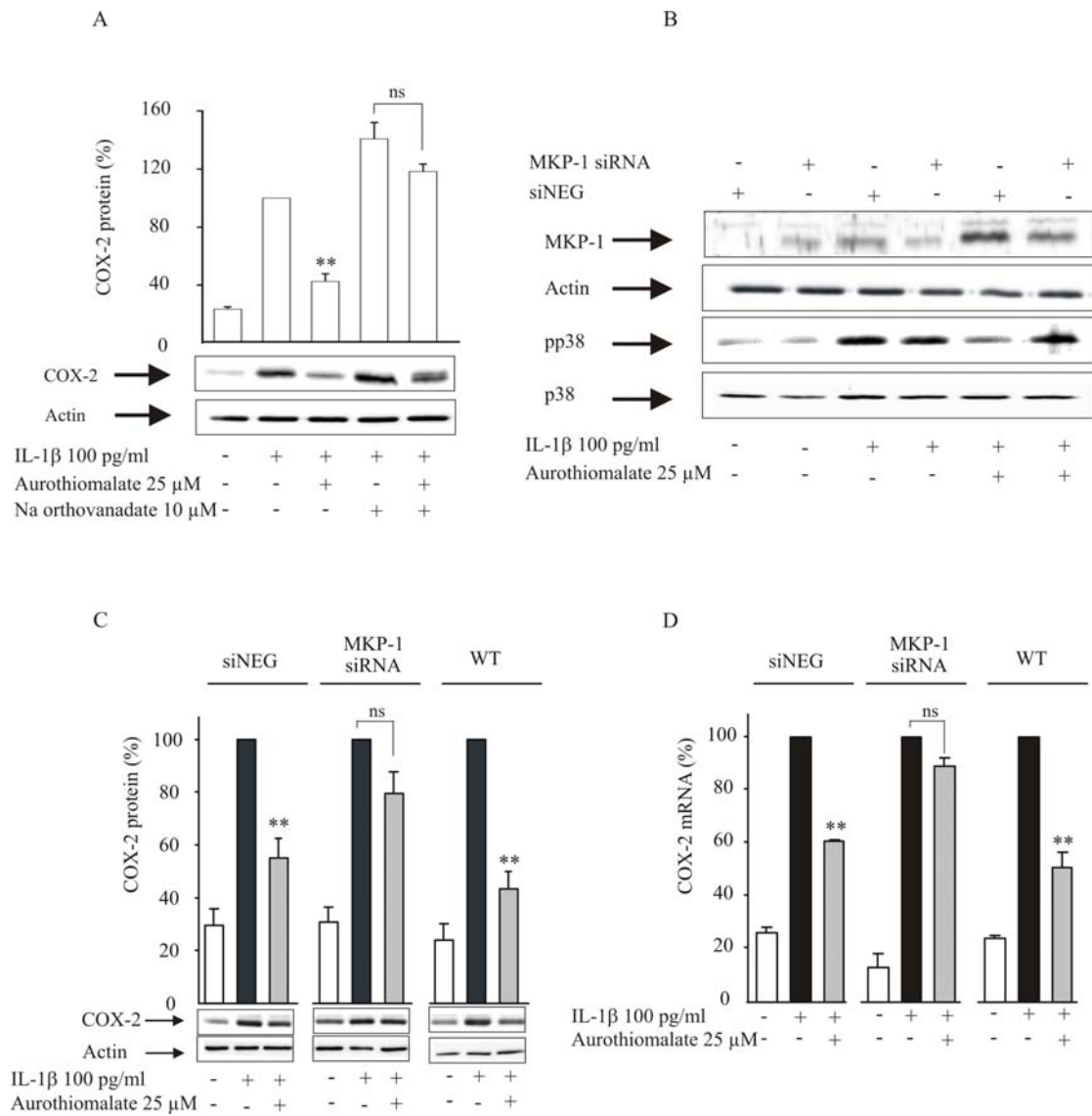
Figure 17. *The effect of aurothiomalate on COX-2 expression in the presence of tyrosine phosphatase inhibitor or when MKP-1 was down-regulated by siRNA. (A) H4 chondrocytes were stimulated with IL-1β (100 pg/ml) and treated with aurothiomalate (25 µM), sodium orthovanadate (10 µM) or with a combination of aurothiomalate (25 µM) and sodium orthovanadate (10 µM). After 24 h, incubations were terminated and COX-2 protein were measured by Western blot.(B,C) H4 chondrocytes were transiently transfected with MKP-1 –specific siRNA and were incubated with IL-1β (100 pg/ml) or with a combination of IL-1β and aurothiomalate (25 µM). Incubations were terminated at indicated timepoints (MKP-1: 1.5 h; p38, pp38: 1 h; COX-2: 24 h) and proteins were measured by Western blot. (D) H4 chondrocytes were transiently transfected with MKP-1 –specific siRNA and were incubated with IL-1β (100 pg/ml) or with a combination of IL-1β and aurothiomalate (25 µM). After 6 h, incubations were terminated and the extracted total RNA was subjected to RT-PCR. COX-2 mRNA levels were normalized against GAPDH. (A-D) Values are mean ± SEM, n=4-6. ** indicates P<0.01 as compared with cells treated with IL-1β alone.*
(C,D) siNEG: cells transfected with negative control (sequence that is not complementary to any known genes); MKP-1 siRNA: cells transfected with MKP-1 siRNA; WT: wild type cells, i.e. non-transfected cells.

In the further experiments, we used cartilage from wild type and MKP-1$^{-/-}$ mice. Interestingly, in mice cartilage samples, aurothiomalate inhibited IL-1β –induced COX-2 expression by 83 % in cartilage from wild type mice whereas in samples from MKP-1$^{-/-}$ mice, aurothiomalate had no inhibitory effect on COX-2 expression (Figure 18).
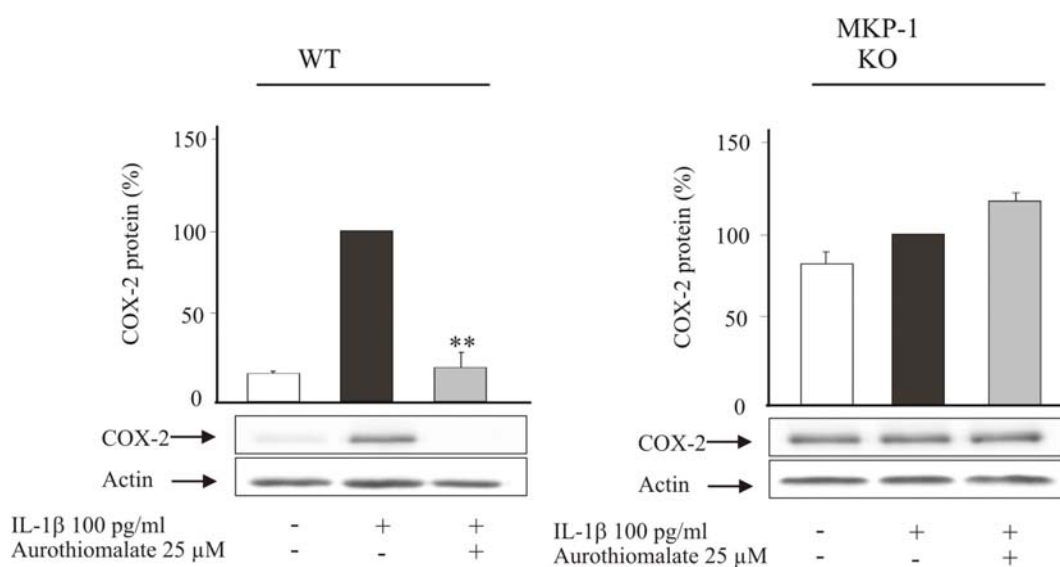

Figure 18. *The effects of IL-1β and aurothiomalate on COX-2 expression in articular cartilage from MKP-1$^{-/-}$ mice. Cartilage samples from wild type (WT) and MKP-1$^{-/-}$ (KO) mice were incubated with IL-1β (100 pg/ml) or with a combination of IL-1β (100 pg/ml) and aurothiomalate (25 μM). After 48 h, incubations were terminated and COX-2 protein was measured by Western blot.*
*Values are mean ± SEM, n=3. ** indicates P<0.01 as compared with cells treated with IL-1β alone.*

In human RA cartilage, aurothiomalate inhibited IL-1β –induced COX-2 protein expression by 58 % (Figure 19A). It was noted that also MMP-3 and IL-6 production was significantly inhibited by aurothiomalate in intact human (Figure 19B,C) and murine (Figure 20C,D) cartilage and in chondrocytes (Figure 20A,B).
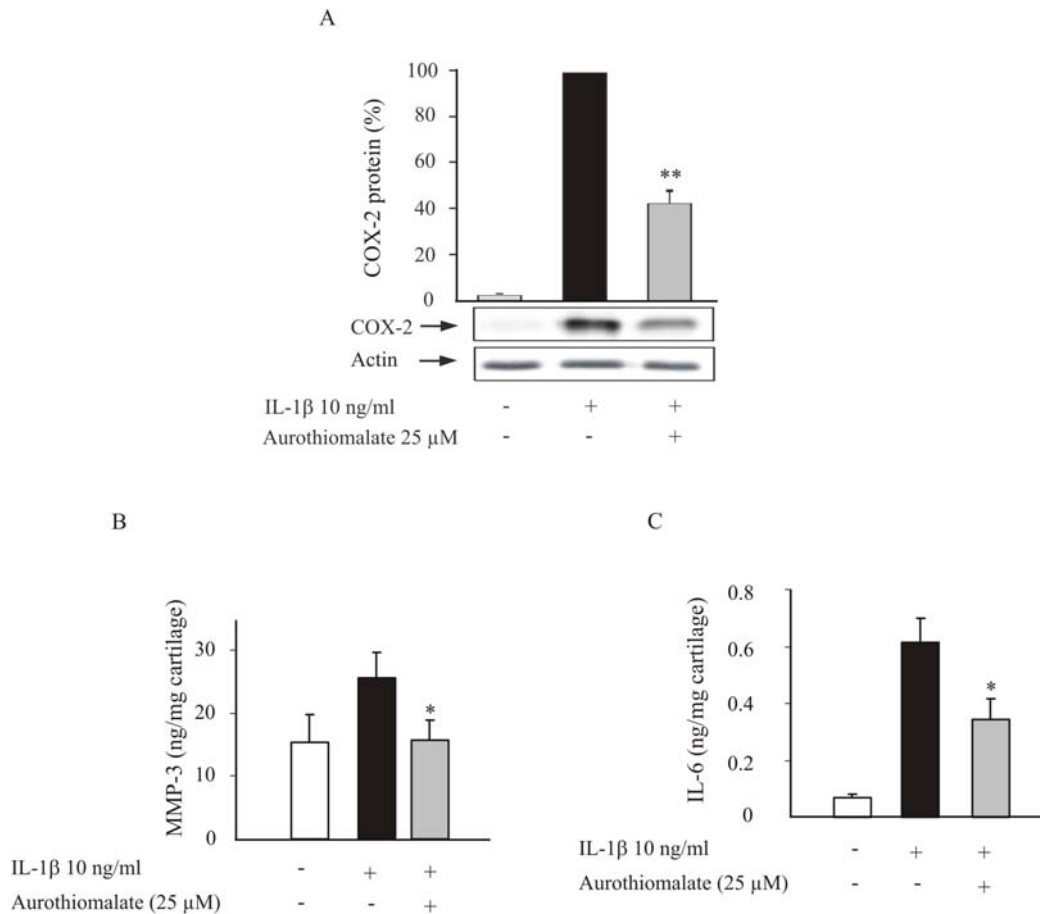

Figure 19. *The effect of aurothiomalate on IL-1β –induced COX-2 expression and MMP-3 and IL-6 production in human cartilage. (A) Human cartilage samples were incubated with IL-1β (10 ng/ml) or with a combination of IL-1β and aurothiomalate (25 μM). After 24 h, incubations were terminated and COX-2 protein was measured by Western blot. (B,C) Human cartilage samples were incubated with IL-1β (10 ng/ml) or with a combination of IL-1β and aurothiomalate (25 μM). After 24 h, incubations were terminated and MMP-3 (B) and IL-6 (C) concentrations were measured in the culture medium by ELISA.*
*Values are mean ± SEM, n=5. ** indicates P<0.05 as compared with cells treated with IL-1β alone.*

Interestingly, not only aurothiomalate's effect on COX-2 seems to be dependent on MKP-1. In the presence of MKP-1 siRNA in chondrocytes or in cartilage

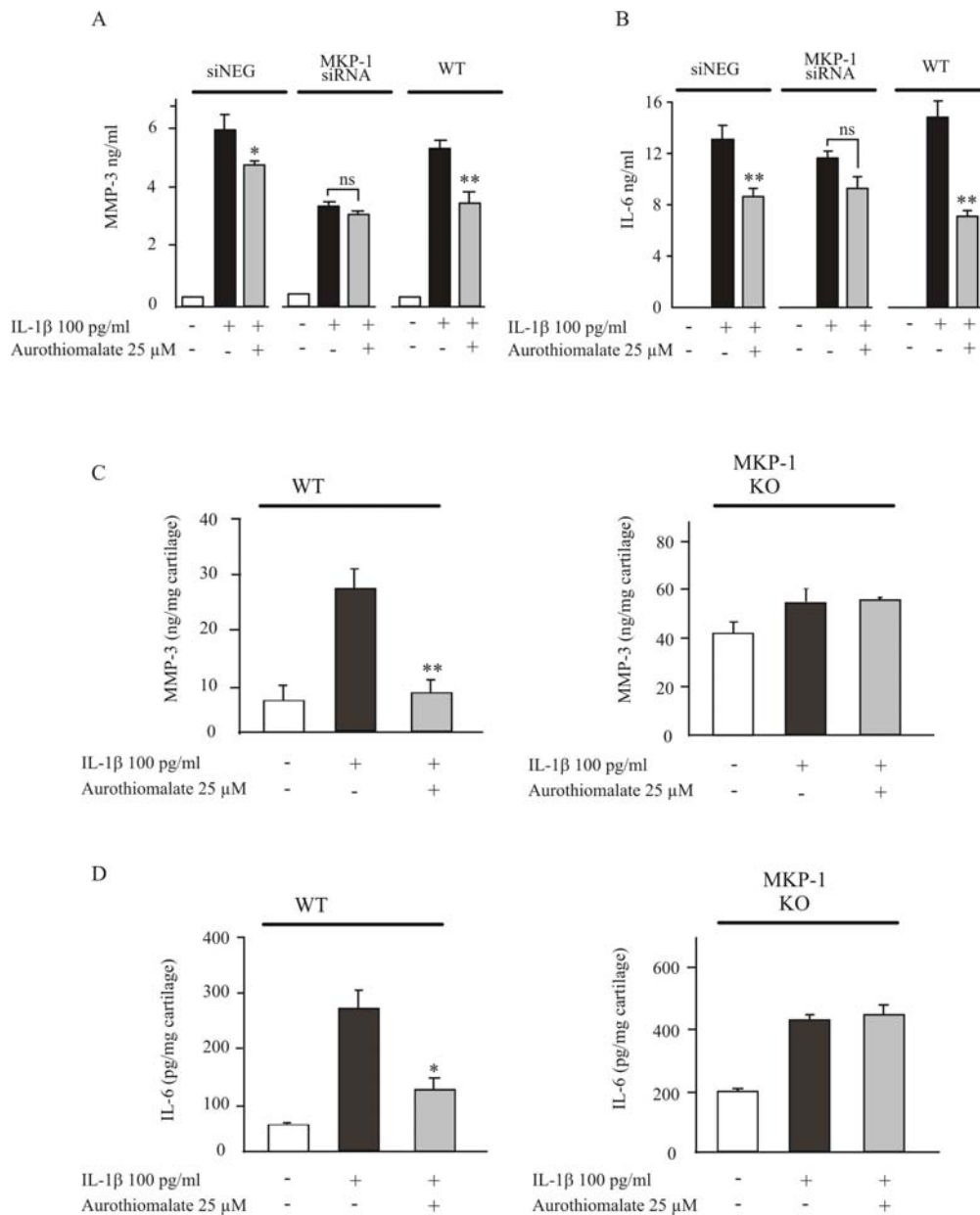samples from MKP-1$^{-/-}$ mice aurothiomalate did not have any effect on MMP-3 or IL-6 production (Figure 20).

Figure 20. *The effect of aurothiomalate on IL-1β –induced MMP-3 and IL-6 production in chondrocytes in which MKP-1 was down-regulated with siRNA, and in cartilage from MKP-1$^{-/-}$ mice. (A,B) Chondrocytes were transfected with MKP-1 –specific siRNA and were incubated with IL-1β (100 pg/ml) or with a combination of IL-1β and aurothiomalate (25 μM). After 24 h, incubations were terminated and MMP-3 (A) and IL-6 (B) production were measured from culture medium by ELISA. (C,D) Cartilage samples from MKP-1$^{-/-}$ (MKP-1 KO) and wild type (WT) mice were incubated with IL-1β (100 pg/ml) or with a combination of IL-1β and aurothiomalate (25 μM). After 48 h, incubations were terminated and MMP-3 (C) and IL-6 (D) concentrations were measured in the culture medium by ELISA. ** indicates $P<0.01$, * indicates $P<0.05$ as compared with cells treated with IL-1β alone.*

Discussion

The study was carried out to investigate the role of MAPK pathways in the regulation of COX-2 expression in chondrocytes. Another major aim was to investigate the effects of DMARDs on COX-2 expression and further to study the mechanism of action of aurothiomalate. MAPK inhibitors were found to inhibit COX-2 expression and subsequent PGE_2 production. Interestingly, the effect of aurothiomalate on COX-2 expression was similar to that seen with inhibitor of p38 MAPK. These results were the impetus to study the effect of aurothiomalate on MAPK pathways. The mechanism behind aurothiomalate's inhibitory action on COX-2 expression was found to be related to its ability to induce MKP-1 expression and reduce p38 MAPK phosphorylation and by that mechanism it was able to inhibit p38 MAPK activity, COX-2 expression and MMP-3 and IL-6 production.

1 Methodology

The study was done in a methodologically progressive manner – starting from immortalized cell lines and chemical inhibitors and ending up with siRNA technology, human cartilage samples and knockout mice.

The use of immortalized cell lines (in this study: human T/C28a2 chondrocytes, murine J774 macrophages, murine H4 chondrocytes) has both advantages and disadvantages. The advantages include a stable, uniform cell population that maintains its properties after several passages and makes the results comparable week after week or even from one year to the next. There are also disadvantages; immortalization often leads to loss of some characteristic features of primary cells and thus the cell lines may not always be true representatives of the primary

cells. With human cartilage samples (obtained from the leftover pieces of knee replacement surgery of OA / RA patients) the greatest advantage is to be allowed to study the reactions of chondrocytes within the cartilage matrix, their natural environment. The disadvantages of these samples include the heterogeneity between samples caused by different disease stage and different patient-related variables that may influence the results. In this study, cartilage tissue samples were obtained also from MKP-1 knockout and wild type mice. The MKP-1 knockout and wild type mice were healthy and age- and sex-matched. In the present study, the results obtained from immortalized cell lines and human and mice cartilage samples were consistent and mutually confirmative.

Standard molecular and cellular biology methods were used to study protein (Western blot) and mRNA (quantitative real-time RT PCR) expression and cytokine (ELISA) and PGE_2 (RIA) production. In Western blot, ELISA and RIA the detection methodology is based on the antigen-antibody –reaction. Laboratory techniques for these methods were as standardized as possible (i.e. in Western blot, the protein concentrations of each sample were measured and the same amount of protein was loaded to each well, and actin was used as an additional loading control of the protein content). All used antibodies were commercial and tested by the manufacturer; the reliability of results depends widely on the antibody's sensitivity and crossreactivity with other proteins. Quantitative real-time RT-PCR was used to measure COX-2 and MKP-1 mRNA in cell culture samples. Housekeeping genes GAPDH and β-actin were used to normalize the RT-PCR results of the genes of interest.

Actinomycin D is an inhibitor of RNA polymerase II and it is widely used as an inhibitor of transcription. With the actinomycin D assay, one can estimate the rate of mRNA decay. In the present study, the actinomycin D assay was applied for measuring COX-2 mRNA stability in H4 chondrocytes. In J774 macrophages, LPS-induced COX-2 mRNA levels were reduced at a slower rate in actinomycin D -treated cells than in control cells. This effect may be due to reduced production of an mRNA degrading factor in the presence of actinomycin D, or due to a more direct (unspecific) stabilizing effect of actinomycin D on

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COX-2 mRNA. Actinomycin D has earlier been shown to inhibit degradation of COX-2 mRNA as well as transferrin receptor mRNA (Seiser et al. 1995, Dixon et al. 2000). These factors should be taken into account when interpreting the results.

MAP kinase inhibitors were used to investigate the role of three different MAPK pathways in the regulation of COX-2 expression and subsequent PGE_2 production. The general weakness in using signaling pathway inhibitors is that compounds may inhibit also other pathways than those desired (Davies et al. 2000, Bain et al. 2003). This should be taken into account when interpreting the results.

The study about the possible mediator role of MKP-1 in aurothiomalate's effect on COX-2 expression was started by using an inhibitor of tyrosine phosphatases (sodium orthovanadate). The results obtained with this non-selective inhibitor were promising and encouraged us to proceed to use MKP-1 siRNA. The siRNA technique is a novel method to down-regulate protein expression (Kong et al. 2007) by using a synthetic siRNA oligonucleotide targeted against the gene of interest. Though it offers many advantages, siRNA method has its own disadvantages as well: transfection efficacy depends on the cell type, transfection reagents may be toxic to cells and siRNAs may have unexpected off-target effects (Aagaard and Rossi 2007). In this study we monitored the transfection efficacy with fluorescent indicator and the down-regulation of the target protein with Western blot, tested toxicity of transfection reagents by XTT test and used a mixture of four siRNAs targeting MKP-1 to minimize off-target effects. Finally, to confirm the results with MKP-1 siRNA, we used inbred C57BL/6 MKP-1 knockout and wild type mice that were originally generated by the R. Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute (Dorfman et al. 1996). The animals were genotyped to monitor the knockout of MKP-1. The benefit in the use of knockout animals compared to siRNA technology is that the gene of interest is totally silenced. In the whole body system knocking out a certain gene may, however, cause compensation by other genes / gene

products which may modify the results and lead to over- or underestimation of the importance of the gene knocked out.

2 The role of MAP kinases in the regulation of COX-2 expression and PGE$_2$ production

In the present study, the p38 inhibitor SB203580, the JNK inhibitor SP600125 and the MKK1/2 inhibitor PD98059 were used as pharmacological tools to study the roles of p38, JNK and Erk1/2 pathways in COX-2 expression and PGE$_2$ production.

The inhibitory effects of SB203580, SP600125 and PD98059 on COX-2 expression and PGE$_2$ production were studied in immortalized human T/C28a2 chondrocytes. Inhibition of the three kinase activities resulted in reduction of PGE$_2$ production and COX-2 expression in a dose-dependent manner. Interestingly, when SB203580 and SP600125 were added 6 h after the challenging agent (IL-1β) they did not inhibit PGE$_2$ production whereas PD98059 had an inhibitory effect also when added at this later time point. This suggested that PD98059 may have some inhibitory effect on COX-2 activity in chondrocytes as has been earlier reported in arachidonic-acid-stimulated human platelets (Borsch-Haubold et al. 1998). According to these results, SB203580 and SP600125 did not inhibit COX-2 activity but instead reduced COX-2 expression.

At mRNA level, SP600125 did not inhibit IL-1β-induced COX-2 mRNA when measured at an early timepoint (2 h). When measured 8 h after IL-1β, SP600125 reduced COX-2 mRNA levels significantly. Further studies of SP600125 were carried out in J774 macrophages. Similar to the results obtained in human chondrocytes, SP600125 inhibited LPS-induced COX-2 expression and PGE$_2$ production in a dose-dependent manner also in J774 macrophages. At the mRNA level, the inhibitory effect of SP600125 on COX-2 mRNA was only seen at a

late timepoint (24 h) whereas there was no effect at the early timepoint (2 h). One would expect a significant inhibition in mRNA levels also at the early timepoints after the stimulus if the regulation was occuring at the transcriptional level. The results with these two cell lines indicated that JNK has only a minor or no effect in the early events of COX-2 transcription and that it rather regulates COX-2 expression at the posttranscriptional level. During the time period 6-12 h, the levels of COX-2 mRNA elevated by LPS declined significantly more rapidly in SP600125-treated cells than in cells treated with LPS only. In the presence of actinomycin D, LPS-induced COX-2 mRNA levels declined much more slowly than in the control cells, and SP600125 had no effect on COX-2 mRNA decay. These results suggested that inhibition of JNK pathway leads to reduced COX-2 mRNA stability or that SP600125 inhibits mechanisms that are related to sustained transcription of COX-2 but are not involved in the early transcriptional activation of COX-2. The inhibitory effect of SP600125 on COX-2 expression has earlier been shown in RAW264 macrophages and in primary rat microglia cultures (Hou et al. 2005, Waetzig et al. 2005). The JNK pathway has been reported to regulate mRNA stability of CLMP, iNOS, IL-2, IL-3 and VEGF (Chen et al. 1998, Ming et al. 1998, Pages et al. 2000, Lahti et al. 2003, Sze et al. 2008). The stabilizing effect of JNK on CLMP and iNOS mRNA may be mediated by an RNA binding protein, TTP (Korhonen et al. 2007, Sze et al. 2008).

In the experiments with SB203580 and PD98059, both compounds inhibited COX-2 mRNA expression in human chondrocytes when measured 2 h after addition of IL-1β and the inhibitory effect was stronger when measured 8 h after addition of IL-1β. The p38 MAPK pathway has been shown to stabilize COX-2 mRNA in mammary carcinoma cells, in HeLa-TO cells and in the human tsT/AC62 chondrocyte cell line (Jang et al. 2000, Lasa et al. 2001, Thomas et al. 2002). Consistently with this, in our further studies we found that SB203580 could reduce COX-2 mRNA stability in H4 chondrocytes.

3 Regulation of COX-2 expression and PGE$_2$ production by aurothiomalate

Out of six tested DMARDs (aurothiomalate, cyclosporin A, hydrxychloroquine, leflunomide, its active metabolite A771726, methotrexate and sulfasalazine) aurothiomalate was the only compound that inhibited COX-2 expression and PGE$_2$ production significantly in H4 chondrocytes. Aurothiomalate was found to inhibit COX-2 expression and PGE$_2$ production also in human OA and RA cartilage. Aurothiomalate has been earlier shown to inhibit PGE$_2$ production in peritoneal macrophages, rat astrocytes and human peripheral blood mononuclear cells (Pistritto et al. 1999, Yamashita et al. 2003). Interestingly, aurothiomalate was found to destabilize COX-2 mRNA (original finding) which may well explain its inhibitory effects on COX-2 expression and PGE$_2$ production in inflammation. Aurothiomalate's inhibitory effect on COX-2 expression and PGE$_2$ production was achieved at clinically relevant drug concentrations. Steady-state serum gold levels during aurohiomalate treatment have been reported to be in the range 8.5-28.5 µM (Gerber et al. 1972), and its concentrations in synovial fluid are similar to those found in plasma (Freyberg et al. 1941).

Aurothiomalate is a gold salt of thiomalic acid. We tested the effects of gold and thiomalic acid separately and noted that gold inhibited COX-2 expression and PGE$_2$ production to the same extent as did aurothiomalate whereas thiomalic acid itself had no effect on COX-2 expression or PGE$_2$ production. These results are consistent with *in vivo* studies which demonstrated that the derivatives of thiomalate without gold had no effect in rat adjuvant arthritis model (Walz et al. 1983).

mRNA stabilizing factor HuR has been reported to bind to 3'-UTR of COX-2 mRNA (Dixon et al. 2000, Sengupta et al. 2003). HuR has been shown to regulate COX-2 mRNA stability in colon cancer cells, mammary epithelial cells and in breast and gastric carcinoma (Dixon et al. 2001, Subbaramaiah et al. 2003, Denkert et al. 2004, Mrena et al. 2005, Denkert et al. 2006). Aurothiomalate

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inhibited IL-1β –induced HuR mRNA levels. This may partly explain aurothiomalate's destabilizing effect on COX-2 mRNA. However, the limitation of this study was that we only studied the effect of aurothiomalate on HuR mRNA levels and were not able to investigate if that was translated to HuR protein.

4 The role of mRNA stability in the regulation of COX-2 expression – significance in the development of new drugs

COX-2 mRNA levels peaked after 4 h in T/C28a2 and H4 chondrocytes and after 6 h in J774 macrophages after stimulus (IL-1β in chondrocyte cell lines and LPS in J774 cells) whereafter the mRNA level declined rapidly in H4 chondrocytes and reached basal level at 8 h whereas in T/C28a2 chondrocytes and in J774 macrophages COX-2 mRNA levels rapidly decreased to about 50 % of the peak level, and remained that high up to the 48 h follow-up. When an inhibitor of transcription, actinomycin D, was added to LPS / IL-1β –treated cells at the 4 h (H4 chondrocytes) or at 6 h (J774 macrophages) timepoints, it did not have any effect on COX-2 mRNA levels compared to cells treated only with the challenging agent. This suggested that there is only marginal or no transcription after mRNA levels have reached the maximum. The JNK inhibitor had no effect on COX-2 mRNA levels when measured at 2 h (T/C28a2 chondrocytes) or 3 h (J774 macrophages) after the stimulus. Timepoints represented a situation where COX-2 mRNA levels were rapidly increasing. In contrast, when measured at later timepoints, 8 h (T/C28a2 chondrocytes) or 24 h (J774 macrophages), the JNK inhibitor had a significant effect on COX-2 mRNA levels suggesting that inhibition of JNK can modify the rate of COX-2 mRNA degradation. The situation with p38 inhibitor was more complicated. In T/C28a2 chondrocytes, the p38 inhibitor inhibited COX-2 mRNA levels both at 2 and 8 hour timepoints suggesting that the p38 pathway may be involved in the cellular events leading to upregulation of COX-2 gene transcription. However, the

inhibitory effect of p38 inhibitor at the 8 hour timepoint was stronger than at 2 hour timepoint suggesting that p38 can regulate COX-2 expression also at the post-transcriptional level. When measured in H4 chondrocytes, the p38 inhibitor did not have any effect on COX-2 mRNA levels when measured 3 h after IL-1β whereas when measured 6 h after IL-1β, the p38 inhibitor inhibited COX-2 mRNA levels significantly indicating that p38 can modify the rate of COX-2 mRNA degradation. In summary, these results suggest that the effect of regulatory pathways may vary depending on the cell line. In addition both JNK inhibitor and p38 inhibitor significantly inhibited COX-2 protein expression and PGE_2 production in all of the cell lines studied. These results highlight the importance of the regulation of COX-2 mRNA stability in the control of COX-2 protein expression and PGE_2 production.

Post-transcriptional regulation of COX-2 expression at the level of mRNA stability seems to be an important mechanism during inflammation. AREs have been shown to regulate gene expression by interacting with different RNA-binding proteins (Dean et al. 2004). Several RNA-binding proteins have been shown to bind the 3'-UTR of COX-2 mRNA: AUF1 (Lasa et al. 2000), β-catenin (Lee and Jeong 2006), HuR (Dixon et al. 2001), TTP (Sawaoka et al. 2003), T-cell intracellular antigen 1 (TIA-1) and TIA related protein (TIAR) (Cok et al. 2004). HuR and β-catenin have been reported to stabilize COX-2 mRNA (Dixon et al. 2001, Lee and Jeong. 2006), TIA-1 and TIAR are claimed to be translational silencers (Gueydan et al. 1999, Piecyk et al. 2000) and TTP and AUF1 are known to destabilize COX-2 mRNA (Lasa et al. 2000, Sawaoka et al. 2003). Interestingly, p38 has been shown to be involved in the upregulation of LPS-induced TTP mRNA and protein expression in several human and murine cell lines (Jalonen et al. 2005, Brook et al. 2006).

Various signaling pathways are involved in the regulation of COX-2 expression at the post-transcriptional level in a cell-type dependent manner (Eberhardt et al. 2007). The p38 MAPK pathway has been shown to stabilize COX-2 mRNA in mammary carcinoma cells, in HeLa-TO cells and in the human tsT/AC62 chondrocyte cell line (Jang et al. 2000, Lasa et al. 2001, Thomas et al. 2002).

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The involvement of MK2 has been shown to be critical in p38-dependent COX-2 mRNA stabilization (Winzen et al. 2004). This was the first study to show that the JNK pathway is involved in the regulation of COX-2 mRNA degradation. PKC has been reported to be involved in the regulation of COX-2 mRNA stability in human mammary epithelial cells, rat intestinal epithelial cells and in rat glomerular mesengial cells (Subbaramaiah et al. 2003, Yu et al. 2003, Doller et al. 2007, Doller et al. 2008, Doller et al. 2009). These signaling pathways act by directly or indirectly regulating the activity, localization or expression of the RNA-binding proteins.

The stability of COX-2 mRNA has been shown to be regulated by various pathophysiological and pharmacological factors. TGF-β, glycogen synthase kinase-3β, IL-1β and Zn^{2+} (Sheng et al. 2000, Tamura et al. 2002a, Harding et al. 2006, Thiel et al. 2006, Wu et al. 2008) have all been reported to increase COX-2 mRNA stability. In addition, taxanes, angiotensin II and gastrin have been shown to be able to stabilize COX-2 mRNA (Subbaramaiah et al. 2003, Doller et al. 2008, Subramaniam et al. 2008, Doller et al. 2009). The mechanism of COX-2 mRNA stabilizing effect for taxanes, angiotensin II and gastrin seems to be related to HuR. This study now adds aurothiomalate to the group of drugs that are reported to destabilize COX-2 mRNA along with dexamethasone and thalidomide (Ristimäki et al. 1996, Lasa et al. 2001, Jin et al. 2007). The effect of aurothiomalate on COX-2 mRNA destabilization was very similar to that of dexamethasone: it was associated with inhibition of p38 MAPK phosphorylation and induction in MKP-1 expression (Lasa et al. 2002).

One interesting new methodological technique to be utilized in the post-transcriptional regulation of COX-2 expression is microRNAs (miRNAs). miRNAs are a novel family of small (~19-22 nt) noncoding RNAs transcribed by the genomes of most metazoa. They differ in sequences but are known to be involved in sequence-specific posttranscriptional regulation by affecting mRNA stability and/or translation. (Nilsen 2007). A report from the group of Dey recently described the interaction of miRNAs and COX-2. During embryo implantation, two uterine miRNAs, mmu-miR-199a* and mmu-miR-101a

interact with the 3'UTR of COX-2 mRNA in mouse uterus leading to its translational repression (Chakrabarty et al. 2007, Daikoku et al. 2008). In colon cancer cell lines, miR-101 has been shown to directly silence COX-2 through a translational mechanism (Strillacci et al. 2008). It would be interesting to examine the effect of miRNAs (miR-146 and miR-155) related to inflammation on COX-2 expression. These two miRNAs have been shown to be inducible by proinflammatory stimuli like IL-1, TNF-α and TLRs. (Sheedy and O'Neill 2008) Those RNA sequences have also been detected in synovial fibroblasts and rheumatoid synovial tissue (Stanczyk et al. 2008).

5 The role of MKP-1 in the regulation of COX-2 expression and MMP-3, IL-6 and PGE$_2$ production

The effect of aurothiomalate on COX-2 mRNA stability was comparable to that of the p38 inhibitor, SB203580. Interestingly, aurothiomalate was found to decrease IL-1β –induced p38 phosphorylation and to increase MKP-1 expression. Aurothiomalate's effect on COX-2 expression was found to be comparable to that reported for dexamethasone. Dexamethasone is believed to destabilize COX-2 mRNA (Ristimäki et al. 1996, Lasa et al. 2001) by increasing expression of MKP-1 and phosphatase-mediated inhibition of p38 MAPK (Lasa et al. 2002). The effect of aurothiomalate on MKP-1 expression seemed to be different from that obtained with the other tested compounds (cyclosporin A, hydroxychloroquine, leflunomide, its active metabolite A771726, methotrexate and sulfasalazine); none of them enhanced MKP-1 expression. The increasing effect of aurothiomalate on MKP-1 expression was found to be due to gold and not thiomalate as was the inhibitory effect on COX-2 expression (see above).

The role of MKP-1 in mediating aurothiomalate's inhibitory effect on COX-2 expression was tested in murine chondrocytes and in MKP-1$^{-/-}$ mice. Firstly, sodium orthovanadate (an inhibitor of phosphotyrosyl phosphatases) was used to inhibit MKP-1 activity and the effect of aurothiomalate on COX-2 expression

was studied in those conditions. Sodium orthovanadate itself had a minor enhancing effect on COX-2 expression and in the presence of sodium orthovanadate, aurothiomalate did not inhibit IL-1β –induced COX-2 expression. Secondly, MKP-1 was down-regulated by siRNA targeted to MKP-1. The treatment with the MKP-1 siRNA reduced IL-1β or IL-1β and aurothiomalate – induced MKP-1 protein expression by over 80 %. In conjunction with this effect, the levels of phosphorylated p38 increased significantly. The inhibitory effect of aurothiomalate on COX-2 expression diminished from 50 % (control cells) to less than 20 % (cells treated with MKP-1 siRNA). Thirdly, the effect of aurothiomalate on COX-2 expression was studied in cartilage samples from MKP-1 knockout and wild type mice. In samples from wild type mice, aurothiomalate inhibited COX-2 expression by more than 80 % whereas there was no inhibitory effect on COX-2 expression in samples from MKP-1 knockout mice.

RA cartilage is known to produce many proinflammatory and destructive factors that are involved in the pathogenesis of arthritis (Feldmann et al. 1996b). Aurothiomalate has been reported to reduce serum IL-6 levels in RA patients (Lacki et al. 1995) and to inhibit IL-6, TNF-α and IL-1β production in mononuclear cells from RA synovial membrane (Yanni et al. 1994). Aurothiomalate has also been shown to suppress IL-8 production in endothelial cells, peripheral blood mononuclear cells (Seitz et al. 1992) and rheumatoid synoviocytes (Loetscher et al. 1994) as well as being able to inhibit IL-12 production and IL-2 receptor expression in T cells (Sfikakis et al. 1993). In THP-1 monocytes, aurothiomalate has been shown to inhibit IL-1β production and caspase-1 activity (Seitz et al. 2003). In addition to the COX-2 – PGE$_2$ – pathway, we studied the effects of aurothiomalate on the production of the proinflammatory cytokine, IL-6, and a cartilage destructive factor, MMP-3, in chondrocytes and in intact cartilage. Aurothiomalate was found to inhibit the production of IL-6 and MMP-3 in human RA and murine cartilage as well as in H4 chondrocytes. In conditions, where MKP-1 was knocked down in H4 chondrocytes by siRNA or in cartilage from MKP-1 deficient mice, aurothiomalate had no inhibitory effect on either IL-6 or MMP-3 production.

Overall these results indicate that it is the aurothiomalate-induced MKP-1 expression that mediates the suppressive effects of this gold compound on the expression of COX-2, MMP-3 and IL-6. Interestingly, MKP-1 has been shown to be an important factor in preventing the development of autoimmunity, especially arthritis. Salojin and co-workers demonstrated that after collagen immunization, MKP-1$^{-/-}$ mice developed more severe arthritis than wild type mice. (Salojin et al. 2006)

MAP kinase inhibitors have been developed for the treatment of inflammatory diseases. Another way to inhibit MAPK pathways is to up-regulate MKP expression. The results of the present study showed that aurothiomalate reduced COX-2, MMP-3 and IL-6 expression by a mechanism related to aurothiomalate's ability to induce MKP-1 expression and reduce p38 phosphorylation and in that way it could inhibit p38 MAPK activity (Figure 21). The results offer a novel mechanism to explain the anti-inflammatory and anti-erosive action of aurothiomalate and indicate that MKP-1 could represent an interesting drug target for the development of new drugs to treat arthritis.

In the future it would be interesting to determine whether the anti-inflammatory effects of aurothiomalate on other cell types (for example macrophages and synovial cells) are also dependent on MKP-1. Other questions to be answered are how the expression of MKP-1 is enhanced by aurothiomalate and whether the enhanced expression of MKP-1 is typical only for gold but extends also to the other molecules that are chemically related to gold. Thus this data introduces MKP-1 as a novel therapeutic target in inflammatory diseases. The understanding of the mechanisms of action of the old DMARD, aurothiomalate, may open new approaches to develop novel anti-inflammatory and anti-erosive compounds that do not have the toxicity problems associated with gold compounds.
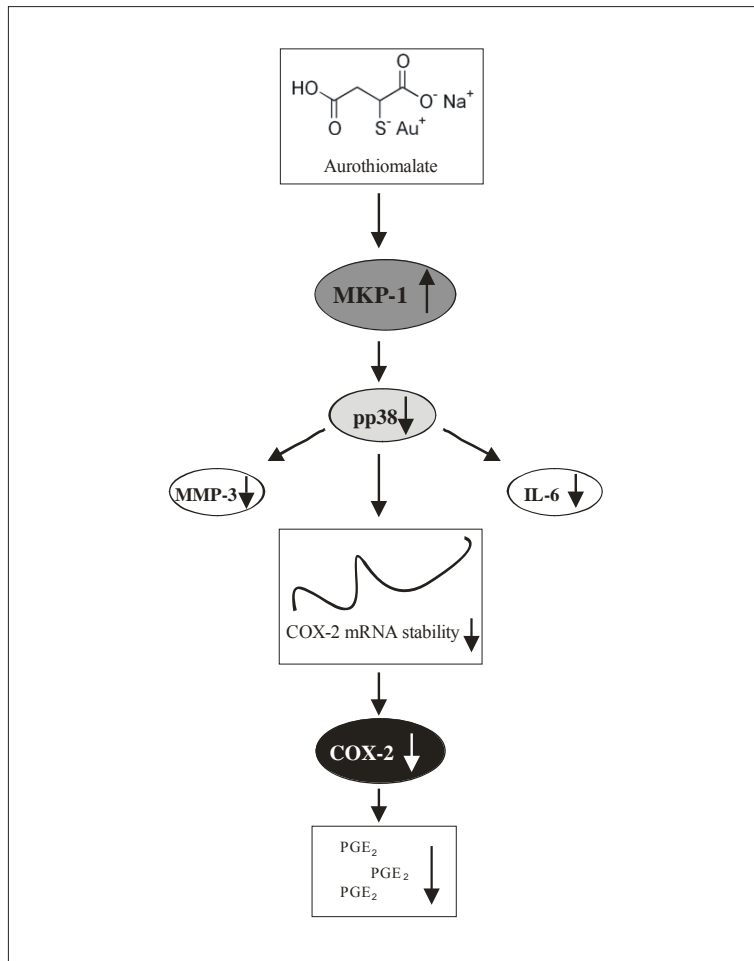
Figure 21. *Summary of the effects of aurothiomalate found in the present study.*

Summary and conclusions

The aim of the present study was to investigate the role of three MAP kinase pathways, i.e. p38, JNK and Erk1/2 in the regulation of COX-2 expression and PGE_2 production in chondrocytes and in intact cartilage as potential targets for development of drugs to combat inflammatory arthritis. Another major aim was to investigate the effects of DMARDs on COX-2 expression and further to study the mechanism of action of aurothiomalate. The major findings and conclusions are as follows:

1. The p38 inhibitor, SB203580, the JNK inhibitor, SP600125 and the Erk1/2 inhibitor, PD98059 all inhibited IL-1β induced COX-2 expression and PGE_2 production in human T/C28a2 chondrocytes. The JNK inhibitor inhibited COX-2 expression and PGE_2 production also in J774 macrophages and p38 inhibitor in H4 chondrocytes by a mechanism that was related to reduced stability of COX-2 mRNA.

2. Aurothiomalate, a traditional DMARD used in the treatment of RA since the 1920s, was found to inhibit COX-2 expression and PGE_2 production in H4 chondrocytes and in human cartilage at concentrations that are clinically achievable. Interestingly, aurothiomalate destabilized COX-2 mRNA in H4 chondrocytes in a similar to the p38 inhibitor, SB203580.

3. In addition to COX-2 expression and PGE_2 production, aurothiomalate was found to reduce MMP-3 and IL-6 production in H4 chondrocytes and in intact human and murine cartilage, and these effects were preceded by increased MKP-1 expression and reduced p38 phosphorylation. Knock-down of MKP-1 by siRNA significantly impaired the ability of aurothiomalate to inhibit the phosphorylation of p38 MAPK and the expression of COX-2, MMP-3 and IL-6. Similarly, aurothiomalate

reduced COX-2, MMP-3 and IL-6 expression in human RA cartilage, and in articular cartilage from wild type mice but not in cartilage from MKP-1$^{-/-}$ mice. These results indicate that it is enhanced MKP-1 expression that is mediating the inhibitory effect of aurothiomalate on the inflammatory and destructive factors COX-2, MMP-3 and IL-6 in cartilage.

MAPK inhibitors are under development for treatment of inflammatory diseases and results from various animal models have shown these agents to have anti-inflammatory properties. Inhibition of COX-2 expression as found in the present study may partially explain the anti-inflammatory effects of the MAPK inhibitors.

Aurothiomalate is known to have both anti-inflammatory and anti-erosive effects in the treatment of RA. Despite a long clinical history in the treatment of RA, the detailed molecular mechanisms behind its anti-inflammatory and anti-erosive action have not been clarified. The findings of the present study provide a novel mechanism to explain the anti-inflammatory and anti-erosive action of aurothiomalate i.e. increased MKP-1 expression, reduced p38 MAPK activation and suppressed expression of COX-2, MMP-3 and IL-6. One further conclusion is that MKP-1 can be concidered as a promising novel target for the development of disease modifying drugs for use in the treatment of inflammatory joint diseases.

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Original communications

Inhibitors of Mitogen-Activated Protein Kinases Downregulate COX-2 Expression in Human Chondrocytes

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Inducible prostaglandin synthase (cyclooxygenase-2, COX-2) is expressed in rheumatoid and osteoarthritic cartilage and produces high amounts of proinflammatory prostanoids in the joint. In the present study we investigated the effects of the inhibitors of mitogen-activated protein kinase (MAPK) pathways Erk1/2, p38, and JNK on COX-2 expression and prostaglandin E_2 (PGE_2) production in human chondrocytes. Proinflammatory cytokine IL-1β caused a transient activation of Erk1/2, p38, and JNK in immortalized human T/C28a2 chondrocytes and that was followed by enhanced COX-2 expression and PGE_2 production. PD98059 (an inhibitor of Erk1/2 pathway) suppressed IL-1-induced COX-2 expression and PGE_2 production in a dose-dependent manner, and seemed to have an inhibitory effect on COX-2 activity. SB203580 (an inhibitor of p38 pathway) but not its negative control compound SB202474 inhibited COX-2 protein and mRNA expression and subsequent PGE_2 synthesis at micromolar drug concentrations. SP600125 (a recently developed JNK inhibitor) but not its negative control compound N^1-methyl-1,9-pyrazolanthrone downregulated COX-2 expression and PGE_2 formation in a dose-dependent manner. SP600125 did not downregulate IL-1-induced COX-2 mRNA expression when measured 2 h after addition of IL-1β but suppressed mRNA levels in the later time points suggesting post-transcriptional regulation. Our results suggest that activation of Erk1/2, p38, and JNK pathways belongs to the signaling cascades that mediate the upregulation of COX-2 expression and PGE_2 production in human chondrocytes exposed to proinflammatory cytokine IL-1β.

INTRODUCTION

Prostaglandins (PGs) are present in a wide variety of human tissues, where they regulate physiological responses, including vascular tone, blood clotting, kidney function, gastric secretion and reproduction [1]. In arthritis, prostaglandins (especially PGE_2) are produced in much higher amounts, and they mediate inflammation, tissue destruction, and inflammatory pain. PGs are synthesized from arachidonic acid by cyclooxygenase (COX) enzymes [2, 3]. Two isoforms of COX have been identified: COX-1 is constitutively expressed and produces low physiological levels of prostanoids, whereas the expression of the inducible isoform, COX-2, is increased in response to proinflammatory cytokines or bacterial products [4]. COX-2 is highly expressed in rheumatoid (RA) and osteoarthritic (OA) cartilage [5, 6]. Interleukin (IL)-1 is a key cytokine involved in the joint destruction in RA and OA and it has been shown to enhance COX-2 expression in articular chondrocytes [5, 7, 8].

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases, that are part of the signal transduction pathways which connect inflammatory and other extracellular signals to intracellular responses, for example, gene expression. The three better characterized MAPK pathways are extracellular signal-regulated kinase 1 and 2 (Erk1/2), p38, and c-Jun N-terminal kinase (JNK). The growth-factor-induced Erk1/2, and the stress-activated p38 and JNK protein kinases are phosphorylated in response to extracellular stimuli at conserved threonine and tyrosine residues and have regulatory functions in inflammation [9, 10]. Inhibitors of p38 and JNK are under development for treatment of arthritis and they

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have shown efficacy in experimentally induced arthritis and joint pain [11, 12]. Inhibition of MAPKs is likely to result in suppression of inflammatory mediators, which in turn leads to the desired therapeutic effects. We hypothesized that one of the inflammatory pathways in the cartilage, that might be down-regulated by MAPK inhibitors, is COX-2-PGE$_2$ pathway. The aim of the present study was to investigate if inhibitors of JNK (SP600125), p38 (SB203580), and Erk1/2 (PD98059) MAP-kinase pathways downregulate IL-induced COX-2 expression and PGE$_2$ production in human chondrocytes.

MATERIALS AND METHODS

Materials

Reagents were obtained as follows: SP600125 (anthra[1,9-*cd*]pyrazol-6(2*H*)-one), SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole), and PD98059 (2-(2′-amino-3′-methoxy-phenyl)-oxanaphthalen-4-one) were from Calbiochem (La Jolla, Calif); goat polyclonal human COX-2, rabbit polyclonal JNK, donkey anti-goat polyclonal, and goat anti-rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc, (Santa Cruz, Calif); and rabbit polyclonal phospho-JNK, phospho-Erk1/2, Erk1/2, phospho-p38, and p38 antibodies were from Cell Signaling Technology, Inc, (Beverly, Mass). All other reagents were from Sigma Chemical Co (St Louis, Mo).

Cell culture

Immortalized human T/C28a2 chondrocytes [13] were grown in Dulbecco's modified Eagle's medium (Cambrex Bioproducts Europe, Verviers, Belgium) and Ham's F-12 medium (Gibco, Paisley, Scotland) (1:1, v/v). Culture media contained 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (all from Gibco, Paisley, Scotland). Cells were seeded on 24-well plates for prostaglandin E$_2$ measurements and on 6-well plates for Western blot and RT-PCR. Cell monolayers were grown for 72 h to confluence before the experiments were started and the compounds of interest were added in fresh medium.

Prostaglandin E$_2$ assays

At the indicated time points, the culture medium was collected for prostaglandin E$_2$ (PGE$_2$) measurement. PGE$_2$ concentrations were determined by radioimmunoassay using reagents from the Institute of Isotopes (Budapest, Hungary).

Western blot analysis

At the indicated time points, cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris base, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride,

2 mM sodium orthovanadate, 10 μg/mL leupeptin, 25 μg/mL aprotinin, 1.25 mM NaF, 1 mM sodium pyrophosphate, and 10 mM *n*-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged (14 500 g for 10 min), and supernatants were mixed in a ratio of 1:4 with SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 5% β-mercaptoethanol) and boiled for 5 min. Protein concentrations in the samples were measured by the Coomassie blue method [14]. After boiling for 5 min, equal aliquots of protein (20 μg) were loaded on a 10% SDS-polyacrylamide electrophoresis gel and electrophoresed for 4 h at 100 V in a buffer containing 95 mM Tris-HCl, 960 mM glycine, and 0.5% SDS. After electrophoresis, the proteins were transferred to Hybond-enhanced chemiluminescence nitrocellulose membrane (Amersham, Buckinghamshire, UK) with semidry blotter at 2.5 mA/cm^2 for 60 min. After transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat milk for 1 h at room temperature and incubated overnight at 4°C with COX-2, JNK, p38, Erk1/2, phospho-specific JNK, phospho-specific p38, or phospho-specific Erk1/2 antibodies in TBS/T containing 5% nonfat milk. Thereafter the membrane was washed 4 times with TBS/T for 5 min, incubated with secondary antibody coupled to horseradish peroxidase in the blocking solution for 0.5 h at room temperature, and washed four times with TBS/T for 5 min. Bound antibody was detected using SuperSignal West Pico chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech Corp, San Leandro, Calif). The quantitation of the chemiluminescent signal was carried out with the use of FluorChem software version 3.1.

RNA extraction and real-time RT-PCR

At the indicated time points, cell monolayers were rapidly washed with ice-cold PBS, and cells were homogenized using QIAshredder (QIAGEN, Valencia, Calif). RNA extraction was carried out with the use of RNeasy kit for isolation of total RNA (QIAGEN). Total RNA (25 ng) was reverse-transcribed to cDNA using TaqMan reverse transcription reagents and random hexamers (Applied Biosystems, Foster City, Calif). cDNA obtained from the RT reaction (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 and concentrations were optimized according to manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and they were 5′ - CAACTCTATATTGCTGGAACATGGA - 3′ (human COX-2 forward primer, 300 nM), 5′-TGGAAGCCTGT-GATACTTTCTGTACT-3′ (human COX-2 reverse primer, 300 nM), 5′-TCCTACCACCAGCAACCCTGCCA-3′ (human COX-2 probe containing 6-FAM as 5′-reporter dye
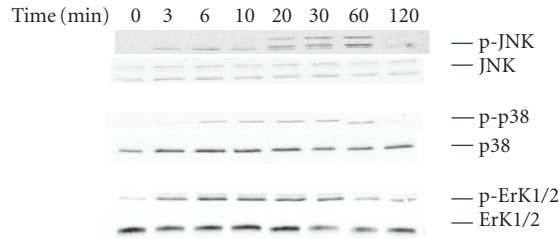
FIGURE 1. The effects of IL-1β on JNK, p38, and Erk1/2 MAPK activation in human T/C28a2 chondrocytes. The chondrocytes were stimulated with IL-1β (100 pg/mL). Incubations were terminated at the indicated time points. Two parallel immunoblots were run from same cell lysates using antibodies against the Thr-183/Tyr-185, Thr-180/Tyr-182, and Thr-202/Tyr-204 phosphorylated (ie, activated) JNK (p-JNK), p38 (p-p38), and Erk1/2 (p-Erk1/2) and against total JNK, p38, and Erk1/2. The experiment was repeated three times with similar results.

and TAMRA as 3′-quencher, 150 nM). Human β-actin was obtained from TaqMan Human β-actin Reagents kit (Applied Biosystems), containing VIC as 5′-reporter dye and TAMRA as 3′-quencher. PCR reaction parameters were as follows: 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 s and annealing and extension at 60°C for 1 min. Each sample was determined in duplicate.

A standard curve method was used to determine the relative mRNA levels as described in the Applied Biosystems User Bulletin: a standard curve for each gene was created using RNA isolated from IL-1β-stimulated human T/C28a2 chondrocytes. Isolated RNA was reverse-transcribed and dilution series of cDNA ranging from 1 pg to 10 ng were subjected to real-time PCR. 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.

Statistics

Results are expressed as the mean ± SEM. Statistical significances were calculated by analyses of variance supported by the Dunnett's multiple comparisons test. Differences were considered significant at $P < .05$.

RESULTS

IL-1β-activated JNK, p38, and Erk1/2 in human T/C28a2 chondrocytes

The ability of IL-1β to activate JNK, p38, and Erk1/2 pathways was studied by Western blot analysis using antibodies directed against Thr-183/Tyr-185, Thr-180/Tyr-182, and Thr-202/Tyr-204 phosphorylated (ie, activated) JNK, p38, and Erk1/2, respectively. JNK activation was seen 20 min after addition of IL-1β. The activation peaked at 1 hour and decreased thereafter. The activation of p38 and Erk1/2 was detected 3–6 min after addition of IL-1β, peaked at 10–20 min, and declined after 1 hour (**Figure 1**).
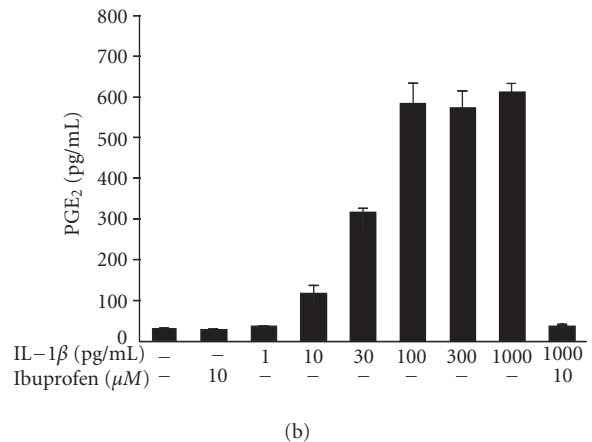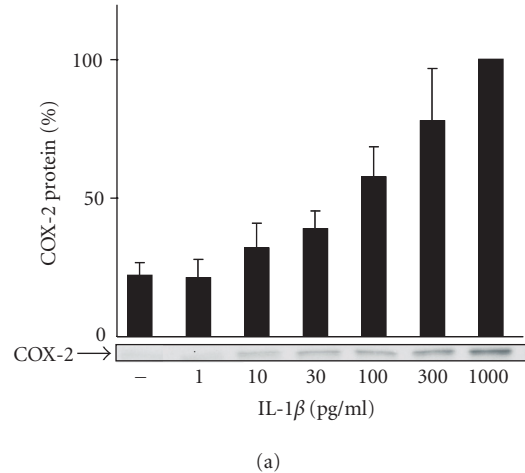

(a)

(b)

FIGURE 2. The effects of IL-1β on COX-2 protein expression and PGE$_2$ production in human T/C28a2 chondrocytes. (a) Human chondrocytes were incubated for 24 h in the presence of increasing concentrations of IL-1β, and COX-2 protein was measured by Western blot. (b) Human chondrocytes were incubated for 24 h in the presence of increasing concentrations of IL-1β, and PGE$_2$ concentrations in the culture medium were measured by radioimmunoassay. COX inhibitor ibuprofen (10 μM) was used as a control compound. Mean ± SEM, $n = 4 - 6$. In (a), a representative gel is shown under the bars.

IL-1β-induced COX-2 expression and PGE$_2$ production in human T/C28a2 chondrocytes

IL-1β enhanced COX-2 expression in a concentration-dependent manner, being detectable at 10 pg/mL and increasing up to 1000 pg/mL (**Figure 2a**). Radioimmunoassay of prostaglandin E$_2$ (PGE$_2$) in the culture medium was carried out to investigate PGE$_2$ production. IL-1β induced PGE$_2$ production in a concentration-dependent manner. Increased PGE$_2$ production was detected at 10 pg/mL of IL-1β and was maximal at 100 pg/mL remaining elevated up to 1000 pg/mL (**Figure 2b**).

SP600125, SB203580, and PD98059 suppressed IL-1β-induced PGE₂ production in human T/C28a2 chondrocytes

Inhibitors of JNK (SP600125), p38 (SB203580), and Erk1/2 (PD98059) reduced IL-1β-induced PGE₂ production in T/C28a2 chondrocytes in a concentration-dependent manner (Figures 3a, 3b, 3c). In the further studies, SP600125, SB203580, and PD98059 were added in T/C28a2 chondrocyte cultures at the same time or 6 h after IL-1β. In contrast to the inhibitory effect seen when added at the same time, SP600125 (10 μM) and SB203580 (1 μM) did not inhibit PGE₂ production when added 6 h after IL-1β. PD98059 (10 μM) inhibited the production of PGE₂ also when added 6 h after IL-1β, but the inhibition was notably less than when added to cells at the same time as the stimulus (Figure 3g).

SP600125, SB203580, and PD98059 inhibited COX-2 expression in human T/C28a2 chondrocytes

In the further studies, we measured the effects of SP600125, SB203580, and PD98059 on IL-1β-induced COX-2 protein expression. Western blots using antibody against COX-2 showed that the three inhibitors caused a concentration-dependent reduction in IL-1β-induced COX-2 protein levels (Figures 3b, 3d, 3f). Negative control compounds were available for SP600125 and SB203580 and their effects on COX-2 expression were also tested. N¹-methyl-substituted pyrazolanthrone is structurally related to SP600125 but it is over 100-fold less potent inhibitor of JNK than SP600125 [15]. N¹-methyl-1,9-pyrazolanthrone (10 μM) had no effect on COX-2 expression while SP600125 (10 μM) reduced COX-2 expression by 70%. SB202474 is structurally related to SB203580 but does not inhibit p38 [16]. SB202474 (1 μM) did not suppress COX-2 expression while SB203580 (1 μM) inhibited COX-2 expression by 40%.

SP600125, SB203580, and PD98059 inhibited COX-2 mRNA expression in human T/C28a2 chondrocytes

We used real-time RT-PCR to investigate the effects of SP600125, SB203580, and PD98059 on COX-2 mRNA expression. IL-1β induced transient COX-2 expression that peaked 4 h after addition of IL-1. SB203580 and PD98059 reduced IL-1β-induced COX-2 mRNA expression significantly when measured either 2 h or 8 h after IL-1β stimulation. In contrast, SP600125 had no marked effect on IL-1β-induced COX-2 mRNA expression at the 2 h time point, whereas the level of COX-2 mRNA was reduced by about 75% at the 8 h time point (Figure 4).

DISCUSSION

In the present study, we found that inhibitors of JNK, p38, and Erk1/2 pathways downregulate IL-1-induced COX-2 expression and PGE₂ production in human chondrocytes.

Consistently with earlier findings using primary articular chondrocytes [17, 18], our results show that IL-1β causes a rapid activation of JNK, p38, and Erk1/2 MAP kinases in immortalized human T/C28a2 chondrocytes. These events were followed by enhanced COX-2 expression and subsequent PGE₂ production. Inhibition of JNK activity by SP600125, p38 activity by SB203580, and Erk1/2 activity by PD98059 resulted in a reduction in the amount of PGE₂ produced. However, when SP600125 and SB203580 were added 6 h after IL-1β, they did not affect PGE₂ production. These findings suggest that SP600125 and SB203580 did not inhibit COX-2 activity, but rather reduced the expression of COX-2. PD98059 inhibited the production of PGE₂ also when added 6 h after IL-1β, but the inhibition was notably smaller than in those experiments where PD98059 was added at the same time as IL-1β. This suggests that PD98059 may have also some inhibitory effect on cyclooxygenase activity in activated chondrocytes, as has been earlier reported in arachidonic-acid-stimulated human platelets [19]. Western blot analysis showed that all the three inhibitors (SP600125, SB203580, and PD98059) caused also a concentration-dependent reduction in COX-2 protein levels in IL-1-treated chondrocytes.

In the real-time RT-PCR studies, SP600125 had practically no effect on IL-1β-induced COX-2 mRNA expression in human T/C28a2 chondrocytes when measured 2 h after IL-1β, whereas when measured 8 h after the addition of IL-1β, a significant reduction in the levels of COX-2 mRNA was observed in the presence of SP600125. These results suggest that inhibition of JNK pathway does not affect the early events in IL-1-induced COX-2 expression but it may regulate the process at post-transcriptional level.

SB203580 and PD98059 had a significant effect on COX-2 mRNA expression when measured 2 h after addition of IL-1β. Our results are consistent with previous reports showing that p38 and Erk1/2 MAP kinase pathways are involved in the cellular events leading to upregulation of COX-2 gene transcription in human monocytes and in RAW264 macrophages [20, 21, 22, 23, 24]. In addition, p38 has also reported to stabilize COX-2 mRNA in mammary carcinoma cells, in HeLa-TO cells, and another chondrocyte cell line [25, 26, 27].

We have earlier shown that, in J774 macrophages, SP600125, SB203580, and PD98059 do not have an effect on nuclear translocation and DNA binding activity of NF-κB [28, 29, 30] which plays a role in stimulating COX-2 expression [31]. In addition to NF-κB, the expression of COX-2 is regulated by other factors including NF-IL6 and AP-2 [32, 33]. Further studies are needed to determine the molecular mechanisms which mediate the effects of JNK, p38, and Erk1/2 inhibitors on COX-2 expression.

Inhibitors of p38 and JNK are under development for treatment of arthritis and they have been shown to have antiinflammatory and antierosive effects in experimentally induced arthritis, and to relief inflammatory pain [10, 11]. The inhibition of MAP kinases and
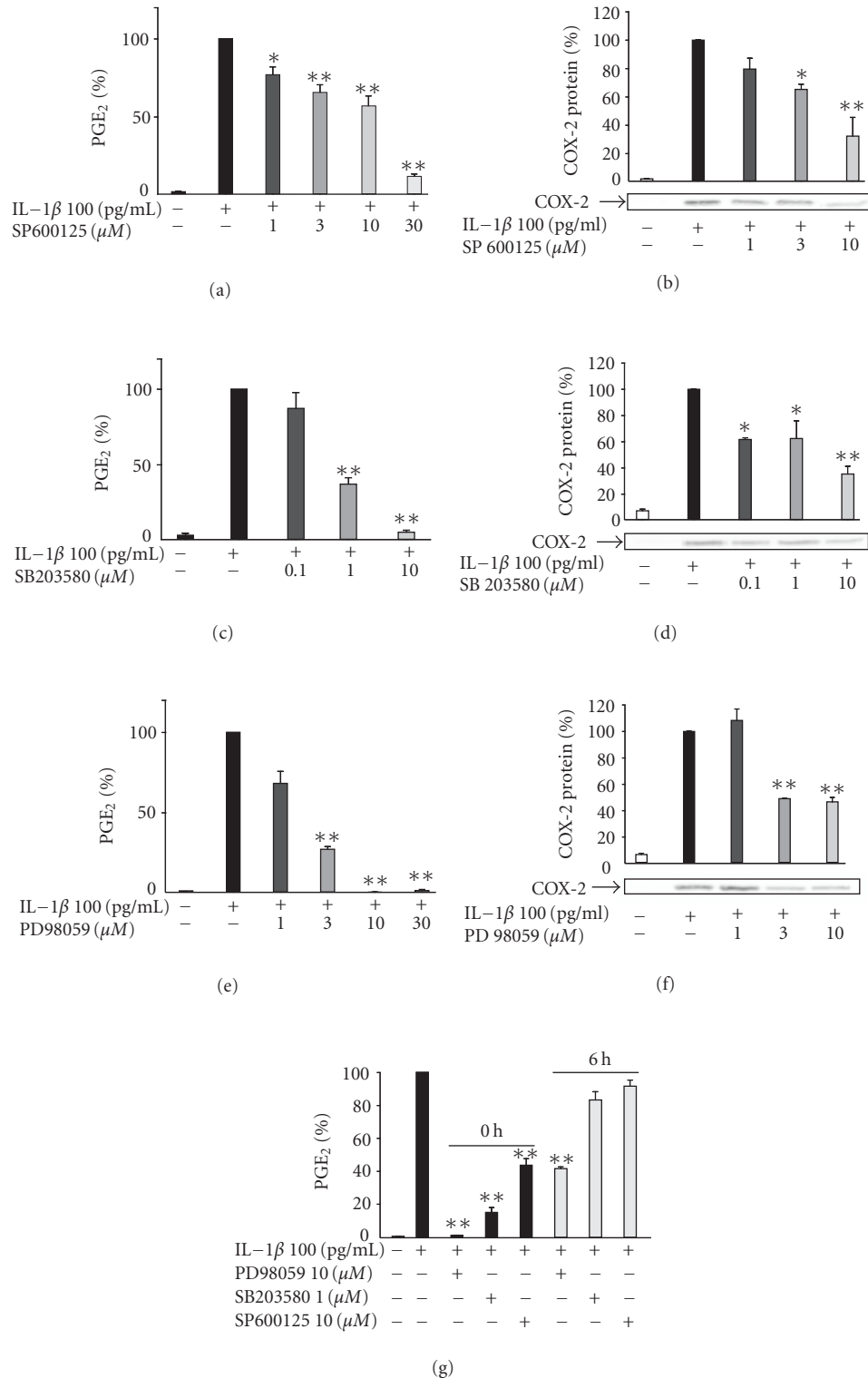
Figure 3. The effects of SP600125, SB203580, and PD98059 on PGE$_2$ production and COX-2 protein expression in IL-1β-stimulated human T/C28a2 chondrocytes. Human chondrocytes were incubated with IL-1β (100 pg/mL) and increasing concentrations of (a), (b) SP600125 (JNK inhibitor), (c), (d) SB203580 (p38 inhibitor), and (e), (f) PD98059 (Erk1/2 inhibitor). After 24 h, incubations were terminated, and PGE$_2$ concentrations in the culture medium were measured by RIA ((a), (c), (e)) and COX-2 protein was measured by Western blot ((b), (d), (f)). (g) SP600125 (10 μM), SB203580 (1 μM), and PD98059 (10 μM) were added to the cell culture at the same time (0 h) or 6 h after IL-1β (6 h). After 24 h, PGE$_2$ concentrations were measured in the culture medium by RIA. Mean ± SEM, $n = 4$, ∗∗ indicates $P < .01$ as compared with the respective control. In (b), (d), and (f) a representative gel is shown under the bars.
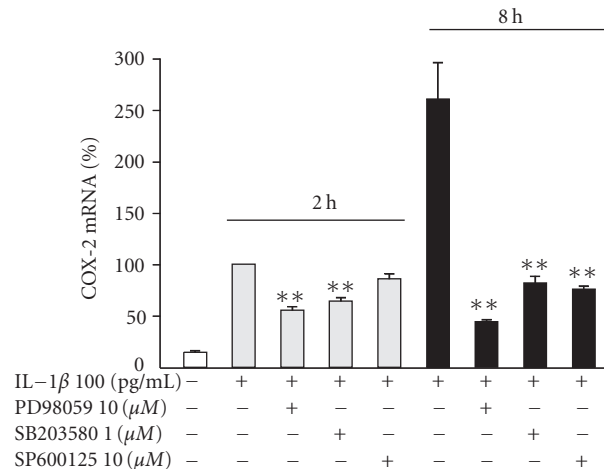
FIGURE 4. The effects of SP600125, SB203580, and PD98059 on COX-2 mRNA levels in IL-1β-stimulated human chondrocytes. Human chondrocytes were incubated with IL-1β (100 pg/ml) and with or without SP600125, SB203580, and PD98059. Incubations were terminated at the indicated time points, and the extracted total RNA was subjected to real-time RT-PCR. COX-2 mRNA levels were normalized against β-actin mRNA. Mean $\pm$ SEM, $n = 6$, $**$ indicates $P < .01$ as compared with cells treated with IL-1β only.

subsequent inhibition of the synthesis of a number of important proinflammatory cytokines like IL-1, TNF-α, IL-6, IL-8, and matrix metalloproteases has been identified as a mechanism contributing to the antiinflammatory activity of these compounds [10, 11, 12, 15, 34, 35]. The present results show that inhibitors of JNK, p38, and Erk1/2 MAP kinases also downregulate COX-2 expression and PGE$_2$ production in human chondrocytes which is likely involved in the mechanisms of their therapeutic effects in arthritis and other inflammatory diseases.

ACKNOWLEDGMENT

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JNK inhibitor SP600125 reduces COX-2 expression by attenuating mRNA in activated murine J774 macrophages

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Abstract

Inducible prostaglandin synthase (cyclooxygenase-2, COX-2) is highly expressed in inflammation. The signaling mechanisms involved in the up-regulation of COX-2 are not known in detail. In the present study we investigated the role of c-Jun NH_2-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family in COX-2 expression and prostaglandin (PG) E_2 production in murine J774 macrophages activated by bacterial lipopolysaccharide (LPS).

LPS caused a transient activation of JNK which was followed by increased COX-2 expression. Anthra(1,9-*cd*)pyrazol-6(2H)-one (SP600125), an inhibitor of JNK, inhibited phosphorylation of c-Jun with an IC_{50} of $5-10$ μM. At the same concentrations SP600125 suppressed also LPS-induced COX-2 protein levels and PGE_2 production. SP600125 did not alter LPS-induced COX-2 mRNA levels when measured 3 h after addition of LPS, whereas mRNA levels were significantly reduced in SP600125-treated cells when measured 24 h after addition of LPS. LPS-induced COX-2 mRNA levels reduced faster in cells treated with SP600125 than in control cells. Cycloheximide (that is known to activate JNK) enhanced COX-2 expression and its effect was inhibited by SP600125.

The present results suggest that JNK pathway is involved in the up-regulation of COX-2 expression possibly by a mechanism related to the stability of COX-2 mRNA.

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Keywords: COX-2; JNK; Macrophage; MAPK; SP600125; PGE_2

1. Introduction

Prostaglandins (PGs) are present in a wide variety of human tissue. PGs play a central role in inflammation and regulate various physiological responses including vascular tone, blood clotting, kidney function, gastric secretion and reproduction [1]. PGs are formed from arachidonic acid by the prostaglandin synthesizing cyclooxygenase (COX) enzymes [2,3]. Two isoforms

of COX have been identified: COX-1 is constitutively expressed and produces low physiological levels of prostanoids, whereas the expression of the inducible isoform, COX-2, is increased e.g. in response to pro-inflammatory cytokines and bacterial products [4].

The c-Jun NH_2-terminal kinase (JNK) belongs to a signaling cascade of mitogen-activated protein kinases (MAPKs) which are a group of serine/threonine kinases that mediate the effects of inflammatory and other extracellular signals to intracellular target molecules [5,6]. JNK has been identified as a stress-activated protein kinase that phosphorylates c-Jun on two sites in the NH_2-terminal domain [7,8]. Certain cytokines and

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cellular stress can activate JNK pathway [9]. JNK activation leads to the phosphorylation of transcription factors (most importantly the c-Jun component of AP-1) and other regulatory cellular proteins [10,11].

The aim of the present study was to investigate if JNK pathway regulates COX-2 expression and PGE_2 production in activated macrophages. We used a recently described inhibitor of JNK (SP600125) [12] as a pharmacological tool to inhibit this MAP-kinase pathway in murine J774 macrophages stimulated by LPS.

2. Materials and methods

2.1. Materials

Reagents were obtained as follows: SP600125 (anthra[1,9] pyrazol-6(2H)-one) and N-methyl-substituted pyrazolanthrone (N^1-methyl-1,9-pyrazolanthrone) were from Calbiochem (La Jolla, CA); goat polyclonal mouse COX-2, rabbit polyclonal c-Jun and JNK, donkey anti-goat polyglonal, and goat anti-rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rabbit polyclonal phospho-JNK (Thr183/Tyr185), and phospho-c-Jun (Ser63) antibodies were from Cell Signaling Technology Inc. (Beverly, MA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

J774 murine macrophages (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium with glutamax-I. Culture media contained 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (all from Gibco, Paisley, Scotland). Cells were seeded on 24-well plates for PGE_2 measurements and on six-well plates for Western blot and RT-PCR assays. Cell monolayers were grown for 72 h to confluence before the experiments were started and the compounds of interest were added in fresh culture medium.

2.3. Prostaglandin E_2 assays

At the indicated time points, the culture medium was collected for PGE_2 measurement. PGE_2 concentrations were determined by radioimmunoassay using reagents from the Institute of Isotopes (Budapest, Hungary).

2.4. Western blot analysis

At the indicated time points, cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris base, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM sodiumorthovanadate, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1.25 mM NaF, 1 mM sodium pyrophosphate and 10 mM n-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged ($14,500 \times g$ for 10 min), and supernatants were mixed in a ratio of 1:4 with SDS loading buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0,025% bromophenol blue, and 5% β-mercaptoethanol) and boiled for 5 min. Protein concentrations in the samples were measured by the Coomassie blue method [13]. After boiling for 5 min, equal aliquots of protein (20 µg) were loaded on a 10% SDS-polyacrylamide electrophoresis gel and electrophoresed for 4 h at 100 V in a buffer containing 95 mM Tris–HCl, 960 mM glycine, and 0,5% SDS. After electrophoresis, the proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Buckinghamshire, UK) with semidry blotter at 2.5 mA/cm^2 for 60 min. After transfer, the membrane was blocked in TBS/T (20 mM Tris–base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat milk for 1 h at room temperature and incubated overnight at 4 °C with COX-2, JNK, phospho-specific JNK, c-Jun or phospho-specific c-Jun antibodies in TBS/T containing 5% nonfat milk. Thereafter the membrane was washed 4× with TBS/T for 5 min, incubated with secondary antibody coupled to horseradish peroxidase in the blocking solution for 0.5 h at room temperature, and washed four times with TBS/T for 5 min. Bound antibody was detected using SuperSignal West Pico chemiluminescent substrate
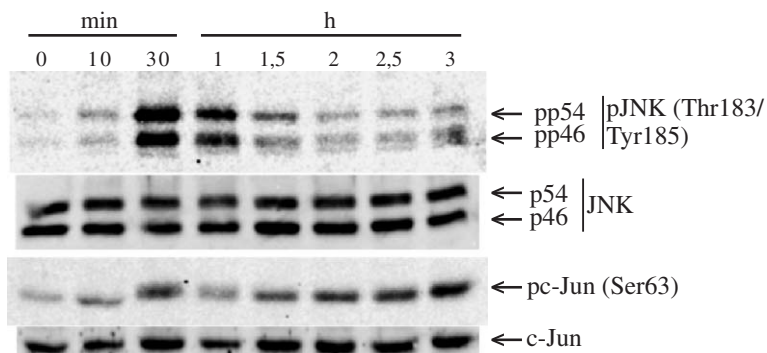

Fig. 1. The effect of LPS on JNK activation. Murine J774 macrophages were stimulated with LPS (1 µg/ml). Incubations were terminated at the indicated time points. Parallel immunoblots were run from same cell lysates using antibodies against the Thr-183/Tyr-185 phosphorylated, i.e. activated JNK (p-JNK), total JNK, Ser63-phosphorylated c-Jun (pc-Jun), and total c-Jun. Data are representative of two experiments with similar results.
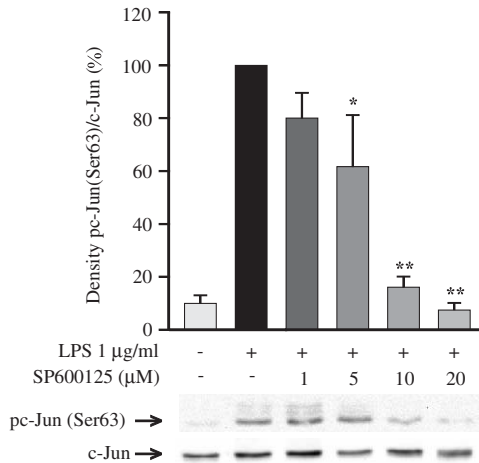
Fig. 2. The effect of SP600125 on JNK activity. Murine J774 macrophages were treated with increasing concentrations of SP600125 for 30 min before addition of LPS (1 µg/ml). Incubations were terminated after 2.5 h, and two parallel immunoblots were run from the same cell lysates using antibodies against Ser63-phosphorylated c-Jun (pc-Jun) and total c-Jun. Phosphorylated c-J un values were normalized to c-Jun values. Mean±S.E.M., $n=4$. *Indicates $P<0.05$, **Indicates $P<0.01$ as compared with cells treated with LPS only.

(Pierce, Cheshire, U.K.) and FluorChem 8800 imaging system (Alpha Innotech Corp., San Leandro, CA). The quantitation of the chemiluminescent signal was carried out with the use of FluorChem software version 3.1.

2.5. RNA extraction and real-time RT-PCR

At the indicated time points, cell monolayers were rapidly washed with ice-cold PBS, and cells were homogenized using QIAshredder (QIAGEN, Valencia, CA). RNA extraction was carried out with the use of RNeasy kit for isolation of total RNA (QIAGEN). Total RNA (25 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA). cDNA obtained from the RT reaction (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 and concentrations were optimized according to manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and were: 5′-GCCAGGGCTGAACTTCGAA-3′ (mouse COX-2 forward primer, 300 nM), 5′-CAATGGGCTGGAAGACA-TATCAA-3′ (mouse COX-2 reverse primer, 300 nM), 5′-CTCACGAGGCCACTGATACCTATTGCATTG-3′ (mouse COX-2 probe, 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher), 5′-GCATGGCCTTCCGTGT-TC-3′ (mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer, 300 nM), 5′-GATGTCATCATACT-TGGCAGGTTT-3′ (mouse GAPDH reverse primer, 300 nM),
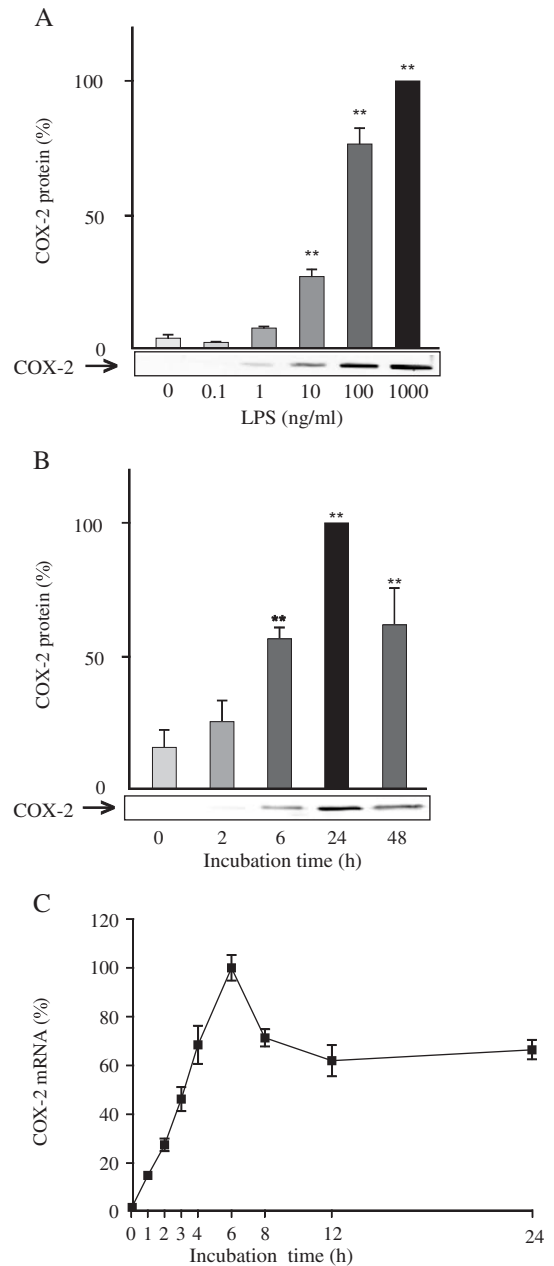

Fig. 3. The effect of LPS on COX-2 expression in J774 macrophages. In A, J774 macrophages were incubated for 24 h in the presence of increasing concentrations of LPS. In B, J774 macrophages were incubated in the presence of LPS (10 ng/ml). Incubations were terminated at the indicated time points. In A and B, COX-2 protein expression was measured by Western blot. In C, J774 macrophages were incubated in the presence of LPS (10 ng/ml). Incubations were terminated at the indicated time points and the extracted total RNA was subjected to RT-PCR. COX-2 mRNA levels were normalized against GAPDH. Mean±S.E.M., $n=3$. **Indicates $P<0.01$ as compared with cells cultured in the absence of LPS.
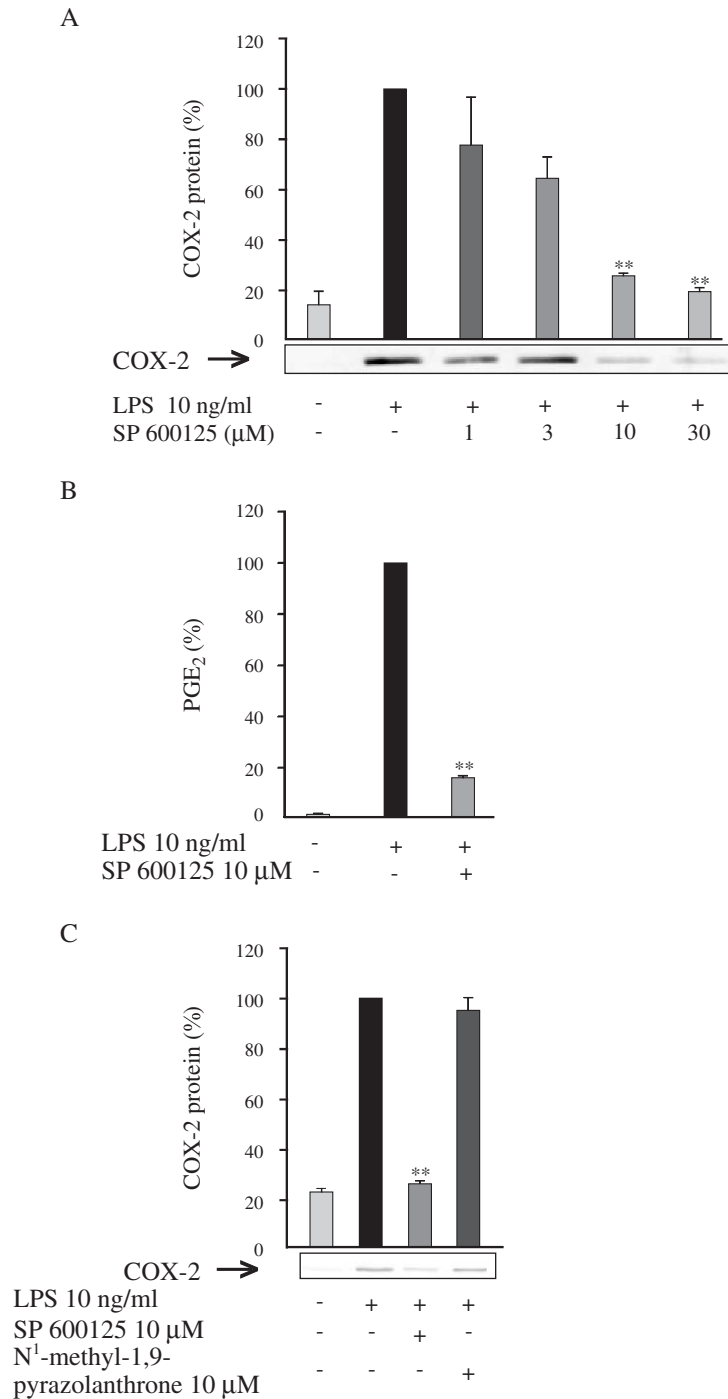
Fig. 4. The effects of SP600125 on COX-2 protein expression and PGE$_2$ production in LPS stimulated macrophages. A, murine macrophages were incubated with LPS (10 ng/ml) and increasing concentrations of SP600125. After 24 h, incubations were terminated, and COX-2 protein was measured by Western blot. B, cells were stimulated with LPS and treated with SP600125 (10 μM). PGE$_2$ concentrations in the culture medium were measured by RIA after 24 h incubation. C, cells were stimulated with LPS (10 ng/ml) and treated with SP600125 (10 μM) or N^1-methyl substituted pyrazolanthrone (negative control compound) (10 μM). After 24 h, incubations were terminated and COX-2 protein was measured by Western blot. Mean±S.E.M., n=4. **Indicates $P<0.01$ as compared with cells treated with LPS only.

5′-TCGTGGATCTGACGTGCCGCC-3′ (mouse GAPDH probe, 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher). PCR reaction parameters were as follows: 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 s and annealing and extension at 60 °C for 1 min. Each sample was determinated in duplicate.

A standard curve method was used to determine the relative mRNA levels as described in the Applied Biosystems User Bulletin: A standard curve for each gene was created using RNA isolated from LPS-stimulated J774 macrophages. Isolated RNA was reverse-transcribed and dilution series of cDNA ranging from 1 pg to 10 ng were subjected to real-time PCR. 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. When calculating the results COX-2 mRNA levels were first normalized against GAPDH.

2.6. Statistics

Results are expressed as the mean±S.E.M. Statistical significances of the differences were calculated by analyses of variance supported by the Dunnett's multiple comparisons test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. LPS activated JNK in J774 macrophages

The ability of LPS to activate JNK pathway was studied by Western blot analysis with an antibody directed against Thr183/Tyr185 phosphorylated JNK. JNK activation increased rapidly after addition of LPS, peaked at 30 min, and declined thereafter (Fig. 1). C-Jun is a direct target of JNK, which phosphorylates c-Jun at residue Ser63 [14]. Levels of Ser63-phosphorylated c-Jun increased in cells after LPS stimulation, and remained elevated up to the 3 h follow-up (Fig. 1).

3.2. SP600125 inhibited LPS-induced c-Jun phosphorylation in J774 macrophages

The ability of SP600125 to inhibit JNK activity in murine J774 macrophages was studied by measuring the phosphorylation of c-Jun residue Ser63 by Western blot. SP600125 inhibited LPS-stimulated Ser63 phosphorylation of c-Jun in a concentration-dependent manner, and the reduction of phosphorylation was 40% at 5 μM concentration and >90% at 20 μM concentration of SP600125 (Fig. 2).

3.3. LPS induced COX-2 expression in J774 macrophages

Western blot analysis of COX-2 protein showed that LPS (10–1000 ng/ml) enhanced COX-2 expression in a concentration-dependent manner in J774 macrophages (Fig. 3A). Fig. 3B and C show the time courses of LPS-induced COX-2 protein and COX-2 mRNA expression, respectively.

3.4. SP600125 inhibited COX-2 expression and PGE₂ production in J774 macrophages

SP600125 reduced LPS-induced COX-2 protein expression in a concentration dependent manner (Fig. 4A). SP600125 had also a clear inhibitory effect on LPS-induced PGE$_2$ production (Fig. 4B). N^1-methyl substituted pyrazolanthrone is a compound structurally related to SP600125 but it is over 100-fold less potent inhibitor of JNK than SP600125 [12] and it was used as a negative control compound in the present study. N^1-methyl-1,9-pyrazolanthrone (10 μM) did not inhibit
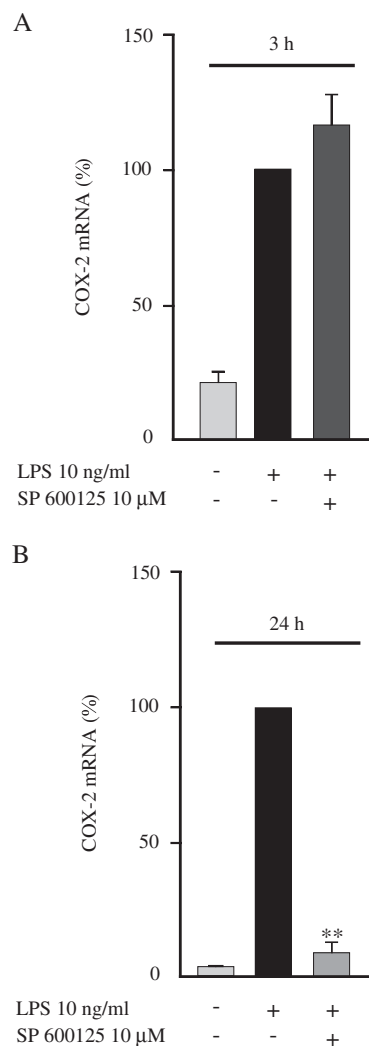

Fig. 5. The effects of SP600125 on COX-2 mRNA expression in LPS stimulated J774 macrophages. J774 macrophages were incubated with LPS (10 ng/ml) with or without SP600125 (10 μM). Incubations were terminated at the indicated time points (A: 3 h, B: 24 h), and the extracted total RNA was subjected to RT-PCR. COX-2 mRNA levels were normalized against GAPDH. Mean±S.E.M., $n=3$. **Indicates $P < 0.01$ as compared with the cells treated with LPS only.

COX-2 expression, whereas SP600125 (10 µM) reduced COX-2 expression by 70% (Fig. 4C).

3.5. SP600125 inhibited COX-2 mRNA expression in J774 macrophages

We used real-time RT-PCR to investigate the effects of SP600125 on LPS induced COX-2 mRNA expression. SP600125 did not inhibit COX-2 mRNA expression when measured 3 h after addition of LPS (Fig. 5A). In contrast, when measured 24 h after LPS, COX-2 mRNA levels were reduced by more than 90% in SP600125-treated cells (Fig. 5B).

In the further studies we measured the effects of SP600125 on COX-2 mRNA degradation by actinomycin D-assay. Cells were treated with LPS or a combination of LPS and SP600125 for 6 h (which represents the peak level of COX-2 mRNA expression see Fig. 3C), and thereafter an inhibitor of transcription, actinomycin D (1 µg/ml) or a solvent control, was added into the culture. Total RNA was extracted before and 2, 4, and 6 h after addition of actinomycin D. In the absence of actinomycin D, COX-2 mRNA levels reduced clearly faster in cells treated with a combination of SP600125 and LPS than in cells treated with LPS alone (Fig. 6A). However, actinomycin D inhibited COX-2 mRNA degradation (Fig. 6B) and SP600125 had no effect in the presence of actinomycin D (Fig. 6C).

3.6. Cycloheximide enhanced COX-2 mRNA expression and JNK activity in LPS-treated J774 macrophages

Cycloheximide has been reported to enhance JNK activity [15]. Western blots of Thr183/Tyr185-phosphorylated JNK and Ser63-phosphorylated c-Jun showed that cycloheximide (0.1 µg/ml) enhanced LPS-induced JNK activation and c-Jun phosphorylation as compared with cells treated with LPS alone. SP600125 markedly inhibited LPS-induced JNK and c-Jun phosphorylation also in cycloheximide-treated cells (Fig. 7A).

To investigate whether cycloheximide would have an opposite effect to that of SP600125, LPS-stimulated J774 cells were treated with cycloheximide, SP600125 and their combination, and RNA was isolated after 12 h incubation. Cycloheximide (0.1 µg/ml) increased LPS-induced COX-2 mRNA expression over 6-fold and SP600125 reduced the effect by 65% (Fig. 7B).
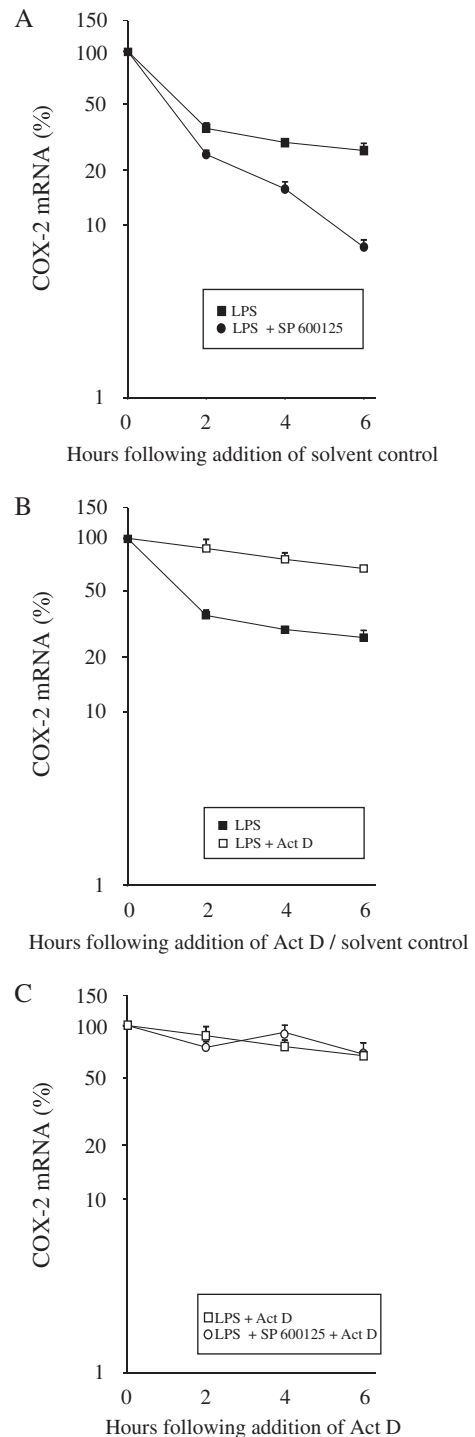

Fig. 6. The effect of SP600125 on COX-2 mRNA half-life in LPS stimulated J774 macrophages. In A, J774 macrophages were incubated with LPS (10 ng/ml) or with a combination of LPS and SP600125 (10 µM). After 6 h solvent control was added into the cell culture. Incubations were terminated at the indicated time points and the extracted total RNA was subjected to RT-PCR. COX-2 mRNA levels were normalized against GAPDH. In B, J774 macrophages were incubated with LPS (10 ng/ml). After 6 h actinomycin D (Act D) or solvent control was added into the cell culture. Incubations were terminated at the indicated time points and the extracted total RNA was subjected to RT-PCR. COX-2 mRNA levels were normalized against GAPDH. In C, J774 macrophages were incubated with LPS (10 ng/ml) or with a combination of LPS and SP600125 (10 µM). After 6 h actinomycin D (Act D) was added into the cell culture. Incubations were terminated at the indicated time points and the extracted total RNA was subjected to RT-PCR. COX-2 mRNA levels were normalized against GAPDH. Mean ± S.E.M., $n = 3$.
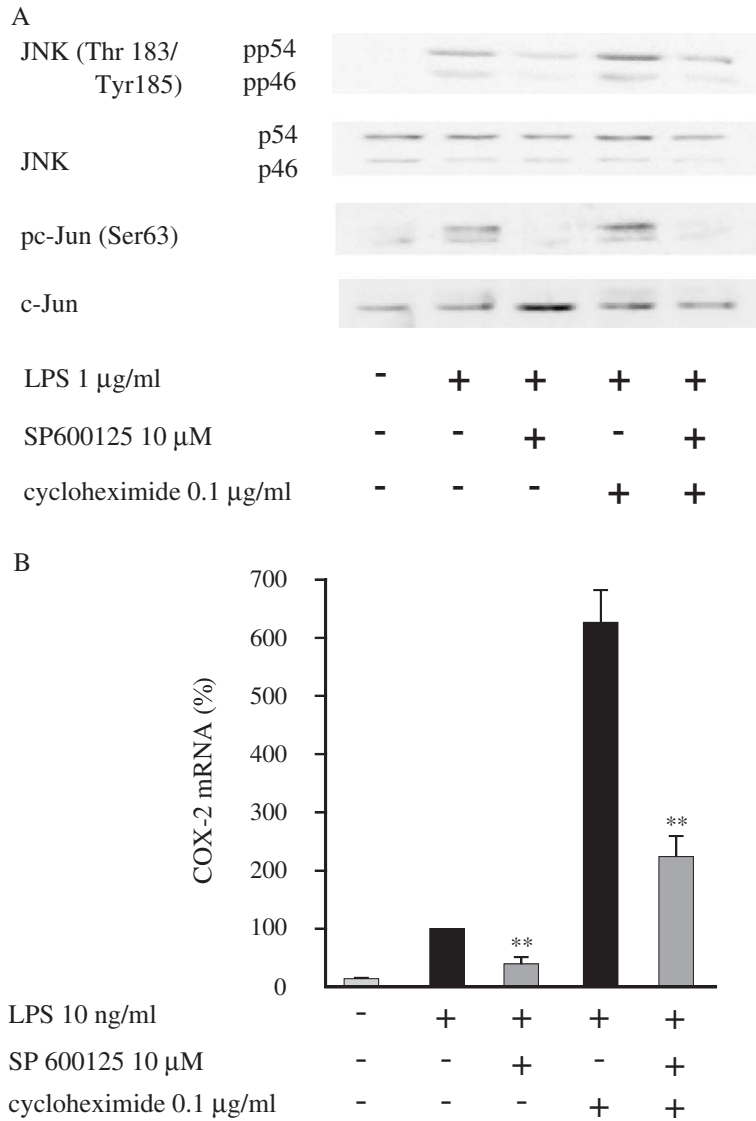
Fig. 7. The effect of cycloheximide on COX-2 mRNA expression and JNK activity. In A, J774 macrophages were treated with SP600125 (10 μM) for 30 min before addition of LPS (1 μg/ml) and cycloheximide (0.1 μg/ml). Incubations were terminated after 30 min (for JNK) and after 2.5 h (for c-Jun), and parallel immunoblots were run from same cell lysates using antibodies against Thr183/Tyr185-phophorylated JNK (pp54 and pp46), total JNK (p54 and p46), Ser63-phosphorylated c-Jun (pc-Jun), and total c-Jun. The results are a representative of three separate experiments with similar results. In B, J774 macrophages were incubated with SP600125 for 30 min before the addition of LPS with and without cycloheximide (0.1 μg/ml). Incubations were terminated after 12 h, and total RNA was isolated. COX-2 and GAPDH mRNA levels were measured by real-time RT-PCR. COX-2 mRNA levels were normalized against GAPDH. Mean ± S.E.M., $n = 6$. **Indicates $P < 0.01$ as compared with the cells treated with LPS only.

3.7. Alsterpaullone and indirubin-3′-monoxime did not inhibit COX-2 expression in J774 macrophages

In addition to its inhibitory effect on JNK, SP600125 has been shown to inhibit cyclin-dependent kinase 2 (CDK2) [16]. CDK2 inhibitors alsterpaullone and indirubin-3′-monoxime have been shown to inhibit CDK2 with IC_{50} values of 15 nM [17] and 0.44 μM [18], respectively. To test whether inhibition of CDK2 would result in reduced COX-2 expression, we treated cells with LPS and CDK2 inhibitors alsterpaullone (50 nM) and indirubin-3′-monoxime (1 μM). Alsterpaullone and indirubin-3′-monoxime did not reduce COX-2 expression (Fig. 8).

4. Discussion

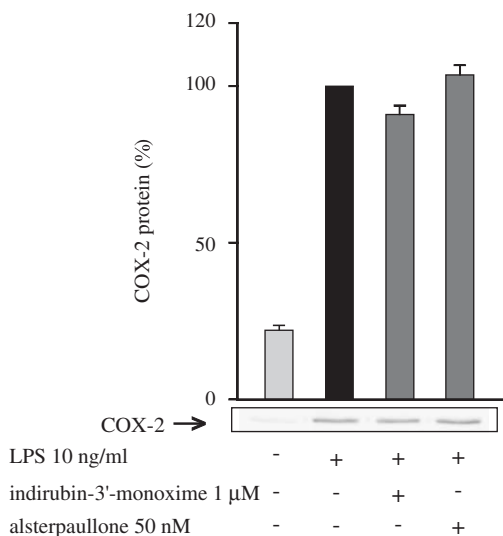In the present study, we found that a JNK inhibitor SP600125 reduced COX-2 expression and PGE$_2$

Fig. 8. The effect of alsterpaullone and indirubin-3′-monoxime on COX-2 expression. Murine macrophages were incubated with LPS (10 ng/ml) and alsterpaullone (50 nM) or indirubin-3′-monoxime (1 μM). After 24 h, incubations were terminated, and COX-2 protein was measured by Western blot. Mean ± S.E.M., $n = 3$.

production in activated J774 macrophages possibly through enhanced COX-2 mRNA decay.

Consistently with earlier findings in Jurkat T cells [12] we found that SP600125 inhibited LPS-induced phosphorylation of c-Jun (which is a target of JNK) with an IC_{50} of 5 to 10 μM in J774 macrophages. SP600125 inhibited also COX-2 expression and PGE_2 production at corresponding drug concentrations. N^1-methyl-1,9-pyrazolanthrone, a negative control compound that is chemically related to SP600125 but does not inhibit JNK at used concentrations [12], did not inhibit COX-2 expression. In addition to JNK, SP600125 has been reported to inhibit cyclin-dependent kinase 2 (CDK2) [16]. Therefore we tested the effects of two other CDK2 inhibitors, alsterpaullone [17] and indirubin-3-monoxime [18] on COX-2 expression. Neither of these compounds inhibited COX-2 expression in our experimental setting. These data suggest that the suppressive effect of SP600125 on COX-2 expression found in the present study is likely to be mediated by its inhibitory effect on JNK.

Cycloheximide is used as a protein synthesis inhibitor, but it is also known to stimulate JNK activity even at concentrations lower than those needed for inhibition of protein synthesis [15]. In the present study cycloheximide (0.1 μg/ml) enhanced JNK activity and COX-2 mRNA expression, and both of these effects were inhibited by SP600125. These data further support

the idea that JNK enhances COX-2 expression in activated macrophages, and that the suppressive action of SP600125 on COX-2 expression is mediated by its inhibitory effect on JNK.

In the present study, SP600125 reduced LPS-induced COX-2 protein expression but it did not inhibit COX-2 mRNA expression when measured 3 h after LPS. In contrast, when measured 24 h after addition of LPS, the reduction of COX-2 mRNA expression was greater than 90% in SP600125-treated cells. These data suggest that SP600125 did not alter the early transcriptional effects of COX-2 in LPS-treated cells. In the further studies we tried to estimate the effects of SP600125 on COX-2 mRNA degradation. LPS induced a transient COX-2 mRNA expression which peaked at 6 h after addition of LPS. LPS-induced COX-2 mRNA levels reduced clearly faster in SP600125-treated cells than in control cells when followed from 6 h to 12 h after addition of LPS. These results point to reduced stability of COX-2 mRNA in the presence of SP600125. Another explanation would be that SP600125 inhibits mechanisms that are related to sustained transcription of COX-2 but are not involved in the early transcriptional activation of COX-2 in response to LPS. In another series of experiments, actinomycin D was added into the cell culture at 6 h after addition of LPS to inhibit transcription. Unexpectedly, LPS-induced COX-2 mRNA levels reduced much slower in actinomycin D-treated cells than in control cells, and SP600125 had no effect on COX-2 mRNA decay in the presence of actinomycin D. The former effect could be due to reduced production of a mRNA degrading factor in the presence of actinomycin D, or due to a more direct stabilizing effect of actinomycin D on COX-2 mRNA (i.e. an effect that is not related to inhibition of transcription). Actinomycin D has also earlier been reported to stabilize COX-2 mRNA as well as transferrin receptor mRNA [19,20]. Taken together, the present data suggest that SP600125 reduced COX-2 mRNA levels in LPS-treated macrophages possibly by destabilizing COX-2 mRNA by a mechanism that is reversed by actinomycin D.

There is increasing body of evidence to support the idea that regulation of COX-2 mRNA stability is a significant mechanism to regulate COX-2 expression. The AU-rich region (ARE) within the 3′-untranslated region (3′-UTR) of COX-2 mRNA has been reported to be important for the regulation of mRNA decay and translation [19,21,22]. Sengupta et al. reported recently that the RNA-binding protein HuR may be an effector molecule that regulates COX-2 expression by binding to and stabilizing ARE-containing regions in COX-2

mRNA [23]. Tristetraproline (TTP) is another protein that binds to AREs and it has been shown to destabilize COX-2 mRNA [24] possibly through a p38-dependent manner [25]. Taxanes seem to increase COX-2 mRNA stability by a mechanism related to HuR [22], whereas dexamethasone has been reported to destabilize COX-2 mRNA [26,27]. In addition, protein kinase C-βII [22,28] and JNK (present study) pathways may be involved in the regulation of COX-2 mRNA stability.

JNK has previously been shown to regulate the expression of iNOS, IL-2, IL-3 and vascular endothelial growth factor (VEGF) by stabilizing their mRNAs [29–32]. Our present findings suggest that JNK regulates also COX-2 expression by decreasing its mRNA decay. In the case of IL-2, the effects of JNK were reported to be mediated via JNK-responsive element (JRE) in the 5′-UTR of IL-2 mRNA. Two RNA-binding proteins, Y box-binding protein and nucleolin, were reported to recognize this element [33]. The detailed mechanism by which JNK mediates its effects on mRNA stability remains to be studied.

SP600125 was introduced as the first small-molecular inhibitor of JNK in primary human CD4 cell culture [12]. It has recently been shown to inhibit COX-2 expression in RAW264 macrophages, primary rat microglia cultures and human chondrocytes [34–36]. In the present study we confirmed that effect in LPS-treated J774 macrophages and provided further support for the idea that the inhibitory effect of SP600125 on COX-2 expression is due to inhibition of JNK activity. In addition, the results show that SP600125 down-regulates LPS-induced COX-2 mRNA levels not at early transcriptional phase but at later time-points following exposure to LPS.

In conclusion, the present results show that SP600125, an inhibitor of JNK, reduces COX-2 expression and PGE_2 production in LPS-treated macrophages possibly by enhancing COX-2 mRNA decay. The results suggest that JNK has an important role in the regulation of COX-2 expression following inflammatory stimuli.

Acknowledgements

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Aurothiomalate inhibits COX-2 expression in chondrocytes and in human cartilage possibly through its effects on COX-2 mRNA stability

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ARTICLE INFO

ABSTRACT

Cyclooxygenase-2 (COX-2) is expressed in rheumatoid and osteoarthritic cartilage and produces pro-inflammatory prostanoids in the joint. In the present study, we investigated the effects of disease modifying anti-rheumatic drugs on COX-2 expression in chondrocytes. Unlike the other tested drugs, aurothiomalate was found to inhibit COX-2 expression in chondrocytes. In the further studies, effects and mechanisms of action of aurothiomalate were investigated in more detail. Aurothiomalate inhibited IL-1β-induced COX-2 protein expression and PGE$_2$ production in chondrocytes in a dose-dependent manner. Because aurothiomalate did not alter IL-1β-induced mRNA levels when measured 0–3 h after addition of IL-1β, its effects on COX-2 mRNA degradation were tested by Actinomycin D assay. The half-life of COX-2 mRNA was reduced from 3 h to less than 1.5 h in aurothiomalate-treated cells. The 3′-untranslated region (3′-UTR) of COX-2 mRNA contains an ARE element which has been shown to bind mRNA stabilizing factor HuR. Interestingly, aurothiomalate inhibited HuR expression which may explain its destabilizing effect on COX-2 mRNA. Aurothiomalate reduced COX-2 expression and PGE$_2$ production also in human cartilage at drug concentrations which have been measured in serum and synovial fluid during treatment with aurothiomalate. The results show that aurothiomalate reduces COX-2 expression and PGE$_2$ production in chondrocyte cultures and in human cartilage. The action is likely mediated by enhanced COX-2 mRNA degradation possibly through a mechanism related to reduced expression of HuR. The results provide a novel mechanism of action for aurothiomalate which may be important in the treatment of arthritis.

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1. Introduction

Prostaglandins are present in a wide variety of human tissues. Prostaglandins play an important role in inflammatory diseases including rheumatoid arthritis and osteoarthritis, and regulate various physiological responses like vascular tone, blood clotting, kidney function, gastric secretion and reproduction (Dubois et al., 1998). Prostaglandins are formed from arachidonic acid which is present in the cellular membranes. Arachidonic acid released from the membranes is metabolized by prostaglandin G/H synthase (cyclooxygenase, COX) to prostanoids (Needleman et al., 1986; Turini and Dubois, 2002). Two isoforms of COX have been identified. COX-1 is constitutively expressed and produces low physiological levels of prostanoids. In an inflammatory focus, the expression of the inducible isoform COX-2 is increased in response to pro-inflammatory cytokines

and bacterial products and it produces prostaglandins, especially prostaglandin E$_2$ (PGE$_2$), which are important mediators of inflammation (Vane et al., 1998; Turini and Dubois, 2002).

In arthritis, COX-2 is expressed in chondrocytes and in the synovial tissue (Amin et al., 1997; Siegle et al., 1998). Pro-inflammatory cytokines IL-1 and TNF-α enhance COX-2 expression and PGE$_2$ production in articular chondrocytes (Geng et al., 1995; Berenbaum et al., 1996; Nieminen et al., 2005). Prostanoids produced by COX-2 pathway can exert catabolic or anabolic effects in the cartilage depending on the microenvironment (Amin et al., 1999; Goldring and Berenbaum 2004). Prostanoids and COX-inhibitors have been reported to regulate collagen production, proteoglycan turnover, and matrix metalloproteinase production in cartilage (Hardy et al., 2002; Fernandez et al., 2004; Goldring and Berenbaum, 2004; Mastbergen et al., 2006). In addition, increased COX-2 expression seems to mediate shear stress-induced chondrocyte apoptosis (Healy et al., 2005). In inflammation, COX-2 expression is regulated both at transcriptional and post-transcriptional level. Depending on the stimulus and the cell type, transcription factors NF-κB, CREB and AP-1 have been proposed

to be important for COX-2 expression (Herschman, 2004), but less is known about the post-transcriptional regulation of COX-2 expression. Regulation of COX-2 mRNA stability may be therapeutically important as dexamethasone has been found to down-regulate COX-2 expression by destabilizing its mRNA (Ristimäki et al., 1996; Lasa et al., 2001). It has been shown that the 3′-untranslated region (3′-UTR) of COX-2 mRNA is able to bind HuR, which is a mRNA stabilizing factor (Dixon et al., 2000; Sengupta et al., 2003). HuR has been reported to be involved in the regulation of COX-2 mRNA stability in colon cancer cells (Dixon et al., 2001; Denkert et al., 2006), mammary epithelial cells (Subbaramaiah et al., 2003) and in breast (Denkert et al., 2004) and gastric (Mrena et al., 2005) carcinoma.

Disease modifying anti-rheumatic drugs suppress inflammation, and retard cartilage degradation and bone erosion in arthritis. The molecular mechanisms of action of many traditional disease modifying anti-rheumatic drugs are not known in detail. Their effects on macrophages and synovial cells have been investigated but very little is known on their effects on cartilage and inflammatory factors produced by affected chondrocytes. In the present study, we investigated the effects of traditional disease modifying anti-rheumatic drugs on COX-2 expression in chondrocytes. Unlike the other tested compounds (cyclosporin A, hydroxychloroquine, leflunomide, its active metabolite A771726 (3-cyano-3-hydroxy-*N*-(4-trifluoromethylphenyl)-crotonamide), methotrexate and sulfasalazine), aurothiomalate was found to be effective at clinically relevant drug concentrations. Therefore, we investigated its effects and mechanisms of action in chondrocyte cultures and in human cartilage in more detail.

2. Materials and methods

2.1. Materials

Reagents were obtained as follows: goat polyclonal mouse COX-2 and actin antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Recombinant human IL-1β was purchased from R&D Systems Inc. (Minneapolis, MA, USA). Hydroxychloroquine and methotrexate were kindly provided by Minna Ruotsalainen, Orion Pharma (Espoo, Finland) and cyclosporin A was supplied by Calbiochem (La Jolla, CA, USA). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture

Immortalized murine H4 chondrocyte cell line developed in the Laboratory of Experimental Rheumatology, University Medical Centre, Nijmegen, The Netherlands (van Beuningen et al., 2002) was used in cell culture experiments. Chondrocytes were grown at 37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Cambrex Bioproducts Europe, Verviers, Belgium) and Ham's F-12 medium (Gibco, Paisley, Scotland, UK) (1:1, v/v). Culture media contained 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (all from Gibco, Paisley, Scotland, UK). Cells were seeded on 24-well plates for prostaglandin E_2 measurements and on six-well plates for Western blot and RT-PCR. Cell monolayers were grown for 48 h to confluence. Thereafter IL-1β and the tested compounds were added in fresh culture medium and incubated for indicated times.

2.3. Cartilage tissue

Cartilage tissue was obtained from the leftover pieces of total knee replacement surgery from patients with osteoarthritis. The study was approved by the ethics committee of Tampere University Hospital and the patients gave their written approval. Full thickness pieces of articular cartilage were removed aseptically from subchondral bone with a scalpel and cut into small pieces. The cartilage samples were incubated at 37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (all from Gibco, Paisley, Scotland, UK).

2.4. XTT test

Cell viability was tested using Cell Proliferation Kit II that measures cells' ability to metabolize sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene sulphonic acid hydrate (XTT) to formazan by mitochondrial dehydrogenase activity that only occurs in viable cells (Boehringer Mannheim, Indianapolis, IN, USA).

2.5. Prostaglandin E_2 assays

At the indicated time points, the culture medium was collected for PGE_2 measurement and kept at −20 °C until assayed. Cartilage tissue pieces were weighed and the results were expressed as ng of PGE_2/g of cartilage tissue. PGE_2 concentrations were determined by radio-immunoassay using reagents from the Institute of Isotopes (Budapest, Hungary).

2.6. Preparation of cell lysates for Western blot analysis

At the indicated time points, cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris–HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenyl-methylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 µM *n*-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged, and supernatants were mixed in a ratio of 1:4 with SDS (sodium dodecyl sulfate) loading buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 5% β-mercaptoethanol) and stored at −20 °C until analyzed. Protein concentrations in the samples were measured by the Coomassie blue method (Bradford, 1976).

2.7. Preparation of cartilage samples for Western blot analysis

After 48 h incubation cartilage was deep frozen in liquid nitrogen and then crushed into powder. Powder was added to cold lysis buffer and samples for Western blot analysis made as described above.

2.8. Western blot analysis

Prior to Western blot analysis, the samples were boiled for 10 min. Equal aliquots of protein (20 µg) were loaded on a 10% SDS–polyacrylamide electrophoresis gel and electrophoresed for 1 h at 120 V in a buffer containing 95 mM Tris–HCl, 960 mM glycine, and 0.5% SDS. After electrophoresis, the proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Buckinghamshire, UK) with semidry blotter at 2.5 mA/cm² for 60 min. After transfer, the membrane was blocked in TBS/T (20 mM Tris–base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat milk for 1 h at room temperature and incubated overnight at 4 °C with COX-2 (SC-1745) or actin (SC-1616) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in TBS/T containing 5% nonfat milk. Thereafter the membrane was washed 4× with TBS/T for 5 min, incubated with secondary antibody (SC-2020) coupled to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in the blocking solution for 0.5 h at room temperature, and washed four times with TBS/T for 5 min. Bound antibody was detected using SuperSignal West Pico chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech Corp., San Leandro, CA, USA). The quantitation of the

chemiluminescent signal was carried out with the use of FluorChem software version 3.1.

2.9. RNA extraction and real-time RT-PCR

Cell homogenization, RNA extraction and reverse transcription of RNA to cDNA were performed as described previously (Nieminen et al., 2006). The primer and probe sequences and concentrations were optimized according to manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and were: 5′-GCCAGGGCTGAACTTCGAA-3′ (mouse COX-2 forward primer, 300 nM), 5′-CAATGGGCTGGAAGACATATCAA-3′ (mouse COX-2 reverse primer, 300 nM), 5′-CTCACGAGGCCACTGATACCTATTGCATTG-3′ (mouse COX-2 probe, 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher), 5′-TGTCCCCGGCAATGCT-3′ (mouse HuR forward primer, 300 nM), 5′-TCACGAATCACTTTCACATTGGT-3′ (mouse HuR reverse primer, 300 nM), 5′-CCTCATCGGCGTCTTGCCCAA-3′ (mouse HuR probe, 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher), 5′-CTCAGAAAGCGGGCGTTGT-3′ (mouse TTP forward primer, 300 nM), 5′-GATTGGCTTGGCGAAGTTCA-3′ (mouse TTP reverse primer, 300 nM), 5′-CCAAGTGCCAGTTTGCTCACGGC-3′ (mouse TTP probe, 200 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher), 5′-GCATGGCCTTCCGTGTTC-3′ (mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer, 300 nM), 5′-GATGTCATCATACTTGGCAGGTTT-3′ (mouse GAPDH reverse primer, 300 nM), 5′-TCGTGGATCTGACGTGCCGCC-3′ (mouse GAPDH probe, 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher). PCR reaction parameters were as follows: 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 s and annealing and extension at 60 °C for 1 min. Each sample was determinated in duplicate.

A standard curve method was used to determine the relative mRNA levels as described in the Applied Biosystems User Bulletin: A standard curve for each gene was created using RNA isolated from IL-1β-stimulated H4 chondrocytes. Isolated RNA was reverse-transcribed and dilution series of cDNA ranging from 1 pg to 10 ng were subjected to real-time PCR. 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. When calculating the results, COX-2, HuR and TTP mRNA levels were first normalized against GAPDH.

2.10. Statistics

Results are expressed as the mean±S.E.M. When appropriate, statistical significances of the differences were calculated by analyses
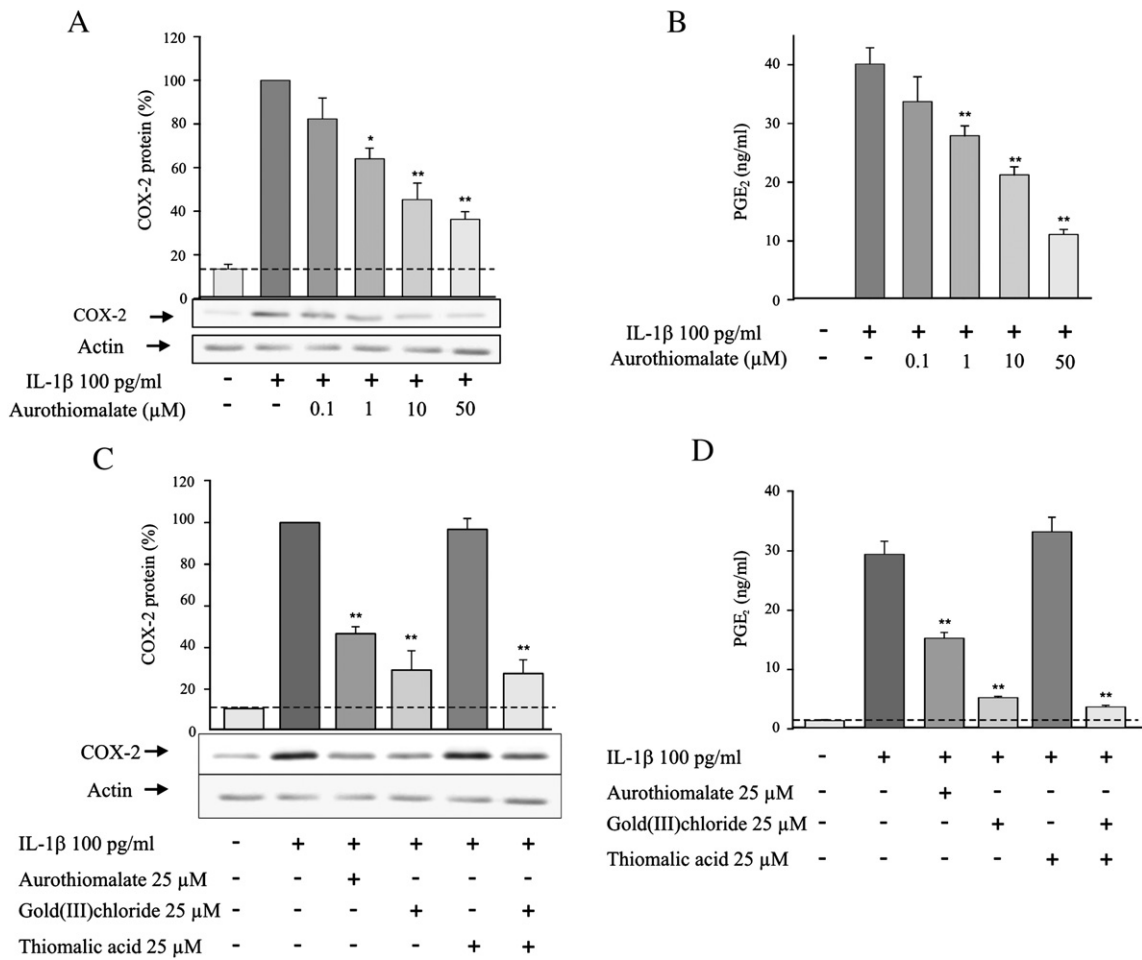

Fig. 1. The effects of aurothiomalate on IL-1β-induced COX-2 protein expression and PGE$_2$ production in chondrocytes. A, H4 chondrocytes were incubated with IL-1β (100 pg/ml) and increasing concentrations of aurothiomalate. After 24 h, incubations were terminated, and COX-2 protein was measured by Western blot. B, cells were incubated with IL-1β (100 pg/ml) and increasing concentrations of aurothiomalate. PGE$_2$ concentrations in the culture medium were measured by radioimmunoassay after 24 h incubation. C and D, cells were stimulated with IL-1β (100 pg/ml) and treated with aurothiomalate (25 μM), gold chloride (25 μM), thiomalic acid (25 μM) or a combination of gold chloride (25 μM) and thiomalic acid (25 μM). After 24 h, incubations were terminated and COX-2 protein was measured by Western blot (C) and PGE$_2$ concentrations in the culture medium were measured by radioimmunoassay (D). Dotted line represents the level of COX-2 expression and PGE$_2$ production in the absence of IL-1β. Mean±S.E.M., $n=3$–4. ** indicates $P<0.01$ as compared with cells treated with IL-1β alone.

of variance supported by the Dunnett's multiple comparisons test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of aurothiomalate, cyclosporin A, hydroxychloroquine, leflunomide, A771726, methotrexate and sulfasalazine on COX-2 expression in H4 chondrocytes

COX-2 expression and PGE$_2$ production were very low in resting cells (Fig. 1A and B). IL-1β (100 pg/ml) enhanced COX-2 protein expression and PGE$_2$ production in H4 chondrocytes. We tested the effects of different disease modifying anti-rheumatic drugs (aurothiomalate, cyclosporin A, hydroxychloroquine, leflunomide, its active metabolite A771726, methotrexate and sulfasalazine; all 10 μM) on COX-2 expression in H4 chondrocytes and found that aurothiomalate inhibited COX-2 expression by 54% whereas other tested drugs had minor or no effect (methotrexate 23% inhibition, A771726 21% inhibition, sulfasalazine 12% inhibition, cyclosporin A 10% inhibition, leflunomide 5% inhibition and hydroxychloroquine no inhibition) (Table 1).

3.2. Aurothiomalate inhibited COX-2 expression and PGE$_2$ production in H4 chondrocytes in a dose-dependent manner

Aurothiomalate reduced COX-2 protein expression in a concentration-dependent manner (0.1 μM: 20% inhibition, 1 μM: 42% inhibition, 10 μM: 63% inhibition and 50 μM: 74% inhibition of IL-1β induced COX-2 expression) (Fig. 1A). Aurothiomalate had also a clear, dose-dependent inhibitory effect on IL-1β-induced PGE$_2$ production (Fig. 1B). In further studies we used aurothiomalate at the concentration of 25 μM which is clinically achievable during treatment with aurothiomalate (Gerber et al., 1972).

In order to investigate whether the effect of aurothiomalate on COX-2 expression and PGE$_2$ production was due to the effect of gold, thiomalate or the combination of them, we studied the effects of gold chloride and thiomalic acid and their combination on COX-2 expression and PGE$_2$ production in H4 chondrocytes. Gold chloride (25 μM) suppressed IL-1β-induced COX-2 expression and PGE$_2$ production by more than 80%, whereas thiomalic acid (25 μM) did not have any effect on COX-2 expression or PGE$_2$ production. The inhibitory effect of the combination of gold chloride and thiomalic acid was similar to that of gold chloride alone which was close to that of aurothiomalate (Fig. 1C and D).

To rule out direct cytotoxic effects, XTT test was used. The test measures cells' mitochondrial dehydrogenase activity that only occurs in viable cells. Triton-X (0.1%) was used as a positive control of cell death. None of the used compounds showed cytotoxic effects when tested in similar conditions where the experiments were carried out (data not shown).

Table 1
Effects of disease modifying anti-rheumatic drugs on IL-1β-induced COX-2 expression in H4 chondrocytes

Compound	Concentration (μM)	COX-2 protein (% of IL-1β induced)
IL-1β		100
+Aurothiomalate	10	46.1 ± 6.8 [a]
+Methotrexate	10	76.8 ± 2.0
+A771726	10	78.9 ± 9.3
+Sulfasalazine	10	87.7 ± 10.7
+Cyclosporin A	10	89.7 ± 17.6
+Leflunomide	10	95.2 ± 1.4
+Hydroxychloroquine	10	100.1 ± 11.6

Cells were incubated for 24 h with the tested disease modifying anti-rheumatic drug (10 μM) and IL-1β (100 pg/ml). COX-2 protein was measured by Western blot. Results are expressed as mean ± S.E.M., $n = 3$.
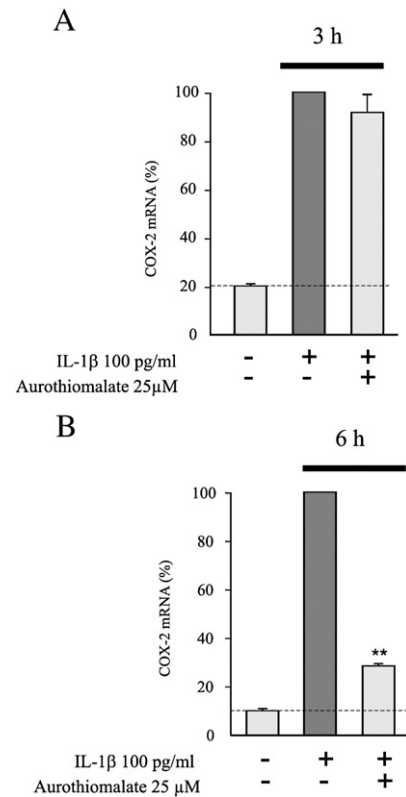[a] Indicates $P < 0.01$ as compared with cells treated with IL-1β alone.

Fig. 2. The effects of aurothiomalate on IL-1β-induced COX-2 mRNA expression in chondrocytes. H4 chondrocytes were incubated with IL-1β (100 pg/ml) or IL-1β (100 pg/ml) and aurothiomalate (25 μM). Incubations were terminated at the indicated time points (A: 3 h, B: 6 h), and the extracted total RNA was subjected to real-time RT-PCR. COX-2 mRNA levels were normalized against GAPDH. The dotted line represents the level of COX-2 mRNA expression in the absence of IL-1β. Mean ± S.E.M., $n = 3$. ** indicates $P < 0.01$ as compared with cells treated with IL-1β alone.

3.3. Aurothiomalate inhibited COX-2 mRNA expression in H4 chondrocytes

We used real-time RT-PCR to investigate the effects of aurothiomalate on IL-1β-induced COX-2 mRNA expression. Aurothiomalate (25 μM) did not inhibit COX-2 mRNA expression when measured 3 h after addition of IL-1β (Fig. 2A). In contrast, when measured after 6 h incubation, IL-1β-induced COX-2 mRNA levels were reduced by about 80% in aurothiomalate-treated cells as compared to the cells cultured with IL-1β in the absence of aurothiomalate (Fig. 2B).
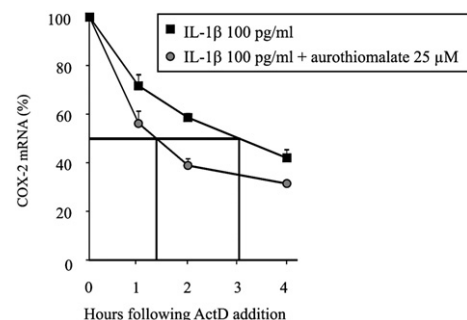

Fig. 3. The effects of aurothiomalate on COX-2 mRNA half-life in IL-1β-stimulated chondrocytes. H4 chondrocytes were incubated with IL-1β (100 pg/ml) or with a combination of IL-1β (100 pg/ml) and aurothiomalate (25 μM). After 4 h incubation actinomycin D (Act D) was added into the cell culture to stop transcription. Incubations were terminated at the indicated time points (1, 2 and 4 h) after addition of Act D and the extracted total RNA was subjected to real-time RT-PCR. COX-2 mRNA levels were normalized against GAPDH. Mean ± S.E.M., $n = 3$.

3.4. Aurothiomalate decreased COX-2 mRNA half-life in H4 chondrocytes

In further studies, we measured the effects of aurothiomalate on COX-2 mRNA degradation by Actinomycin D assay. H4 chondrocytes were exposed to IL-1β or a combination of IL-1β and aurothiomalate for 4 h (which represents the peak level of COX-2 mRNA expression in response to IL-1β in these cells), and thereafter an inhibitor of transcription, actinomycin D (1 μg/ml) or a solvent control, was added into the culture. Total RNA was extracted before and 1, 2 and 4 h after addition of actinomycin D. Real-time RT-PCR analysis showed that when transcription was blocked with actinomycin D, the levels of COX-2 mRNA decreased faster in aurothiomalate-treated cells than in untreated cells (Fig. 3). The half-life of COX-2 mRNA was approximately 3 h in cells treated with IL-1β only, but it was reduced to less than 1.5 h in cells treated with a combination of IL-1β and aurothiomalate.

3.5. Aurothiomalate reduced HuR mRNA levels but did not have an effect on TTP mRNA in H4 chondrocytes

The 3′-untranslated region (3′-UTR) of COX-2 mRNA contains an ARE element which has been shown to bind regulatory protein HuR which stabilizes COX-2 mRNA (Dixon et al., 2001; Katsanou et al., 2005). We investigated whether aurothiomalate has an effect on HuR mRNA expression. Aurothiomalate inhibited HuR mRNA expression when measured 2 and 6 h after addition of IL-1β (Fig. 4A). At 6 h time point aurothiomalate reversed totally the IL-1β-induced HuR mRNA expression. Tristetraproline (TTP) is another ARE-binding protein, and it has been shown to destabilize COX-2 mRNA (Sawaoka et al., 2003). Aurothiomalate had no effect on IL-1β-induced TTP mRNA expression (Fig. 4B).
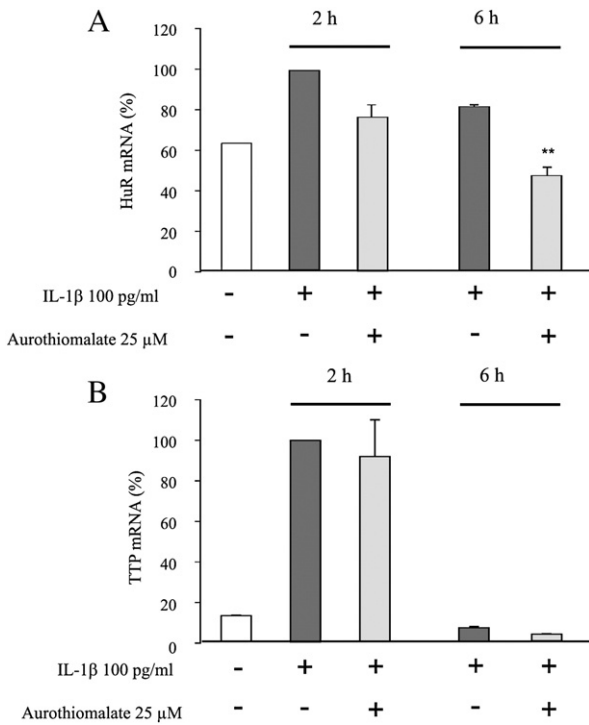

Fig. 4. The effects of aurothiomalate on IL-1β-induced HuR and TTP mRNA expression in chondrocytes. H4 chondrocytes were incubated with IL-1β (100 pg/ml) or a combination of IL-1β (100 pg/ml) and aurothiomalate (25 μM). Incubations were terminated at the indicated time points (after 2 h or 6 h), and the extracted total RNA was subjected to real-time RT-PCR. HuR (A) and TTP (B) mRNA levels were measured and normalized against GAPDH. Mean±S.E.M., $n=3$. ** indicates $P<0.01$ as compared with cells treated with IL-1β alone.
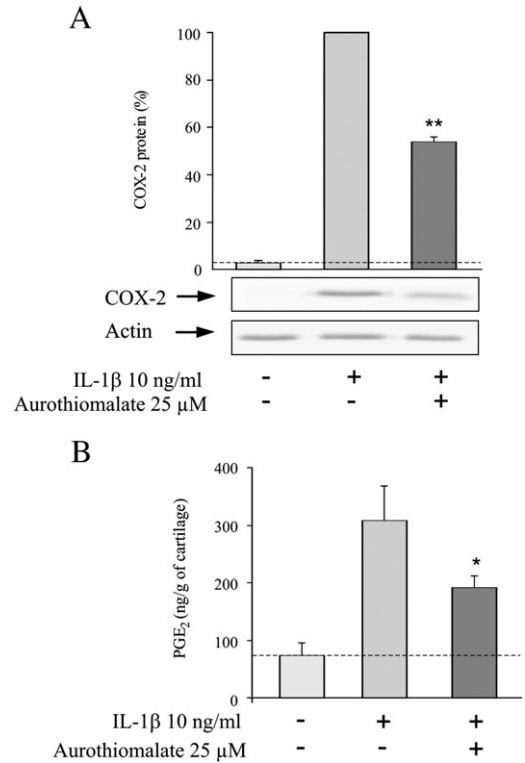

Fig. 5. The effects of aurothiomalate on IL-1β-induced COX-2 protein expression and PGE$_2$ production in human cartilage. Cartilage samples were incubated with IL-1β (10 ng/ml) or a combination of IL-1β (10 ng/ml) and aurothiomalate (25 μM). After 48 h, incubations were terminated, and COX-2 protein was measured by Western blot (A), and PGE$_2$ concentrations in the culture medium were measured by radioimmunoassay (B). The dotted line represents the level of COX-2 expression and PGE$_2$ production in the absence of IL-1β. Mean±S.E.M., $n=5$–6. ** indicates $P<0.01$ as compared with cells treated with IL-1β alone.

3.6. Aurothiomalate inhibited COX-2 expression and PGE$_2$ production in human cartilage

To study the clinical relevance of the finding, we investigated the effects of aurothiomalate on COX-2 expression and PGE$_2$ production in human cartilage. Because of the limited amount of the tissue, only one drug concentration could be tested. The aurothiomalate concentration of 25 μM was selected for the experiments on the basis of the knowledge of the pharmacokinetics of aurothiomalate (Gerber et al., 1972; Freyberg et al., 1941). Aurothiomalate (25 μM) reduced IL-1β-induced COX-2 protein expression (Fig. 5A) and PGE$_2$ production by about 50% (Fig. 5B) in human cartilage.

4. Discussion

In the present study, we investigated the effects of traditional disease modifying anti-rheumatic drugs on COX-2 expression in chondrocytes. Unlike cyclosporin A, hydroxychloroquine, leflunomide, its active metabolite A771726, methotrexate and sulfasalazine, aurothiomalate was effective at clinically relevant drug concentrations, and its effects and mechanisms of action were investigated in more detail. We found that aurothiomalate reduced COX-2 expression and PGE$_2$ production in activated H4 chondrocytes and in human cartilage, and the mechanism of action was related to reduced COX-2 mRNA stability and HuR expression.

Consistently with earlier findings in peritoneal macrophages (Yamashita et al., 2003), rat astrocytes (Pistritto et al., 1999) and human peripheral blood mononuclear cells (Ohta et al., 1986), we found that aurothiomalate inhibited PGE$_2$ production in chondrocyte cultures and in human cartilage. Whereas aurothiomalate seems not

to inhibit PGE$_2$ production in human polymorphonuclear leucocytes (Parente et al., 1986; Moilanen et al., 1988). The inhibitory effect on PGE$_2$ production has been supposed to be involved in the anti-inflammatory effects of aurothiomalate (Vargas et al., 1987) but the mechanisms mediating its suppressive effects on PGE$_2$ production were not known. In the present study, aurothiomalate inhibited COX-2 expression and PGE$_2$ production in chondrocyte cultures and in human cartilage at drug concentrations that are clinically achievable. The steady-state serum gold levels during aurothiomalate treatment have been reported to be 8.5–28.5 μM (Gerber et al., 1972), and its concentrations in synovial fluid are related to those found in plasma (Freyberg et al., 1941).

Aurothiomalate is a gold salt of thiomalic acid. In order to investigate whether the effects of aurothiomalate on COX-2 expression and PGE$_2$ production were due to the effect of gold, thiomalate or the combination of them, we studied the effects of gold chloride and thiomalic acid and their combination on COX-2 expression and PGE$_2$ production. Our results showed that gold chloride suppressed IL-1β-induced COX-2 expression and PGE$_2$ production, whereas thiomalic acid had no effect. The inhibitory effect of the combination of gold chloride and thiomalic acid was related to that of gold chloride alone and that of aurothiomalate. This is in line with previous findings showing that the derivatives of thiomalate without gold had no effect in rat adjuvant arthritis (Walz et al., 1983). Gold chloride has previously been shown to inhibit also TNF-α, IL-1β and IL-6 production in LPS-treated human THP-1 monocytes (Stern et al., 2005).

In the present study, aurothiomalate reduced IL-1β-induced COX-2 protein expression but did not inhibit COX-2 mRNA levels when measured 3 h after addition of IL-1β. In contrast, when measured 6 h after addition of IL-1β, aurothiomalate reduced IL-1β-induced mRNA expression by about 80%. This suggested that aurothiomalate treatment facilitated COX-2 mRNA degradation, and this hypothesis was tested by the mRNA degradation assay. Actinomycin D, an inhibitor of transcription, was added to the cells 4 h after IL-1β which represents the peak level of IL-1β-induced COX-2 mRNA expression in these cells. The half-life of COX-2 mRNA was reduced from 3 h to less than 1.5 h in cells treated with aurothiomalate. To our knowledge, the destabilizing effect of aurothiomalate on COX-2 mRNA has not been reported earlier, and that mechanism could well explain its inhibitory effects on COX-2 expression and PGE$_2$ production in inflammation.

Post-transcriptional regulation of COX-2 expression at the level of mRNA stability seems to be an important mechanism during inflammation. It has been shown that the 3′-untranslated region (3′-UTR) of COX-2 mRNA is able to bind HuR, which is a mRNA stabilizing factor (Dixon et al., 2000; Sengupta et al., 2003). HuR belongs to a family of ELAV (embryonic-lethal abnormal vision)-like proteins and it is ubiquitously expressed (Good, 1997; Nabors et al., 1998). HuR has been reported to be involved in the regulation of COX-2 mRNA stability in colon cancer cells (Dixon et al., 2001; Denkert et al., 2006), mammary epithelial cells (Subbaramaiah et al., 2003) and in breast (Denkert et al., 2004) and gastric (Mrena et al., 2005) carcinoma. Because aurothiomalate reduced COX-2 mRNA stability, we measured its effects on HuR mRNA levels. Interestingly, aurothiomalate inhibited HuR mRNA levels when measured 2 and 6 h after addition of IL-1β, and at the 6 h time point, aurothiomalate totally reversed IL-1β-induced HuR mRNA expression. Inhibition of HuR expression may explain at least in part aurothiomalate's ability to destabilize COX-2 mRNA. Tristetraproline (TTP) is another protein that binds to AREs, and it has been shown to destabilize COX-2 mRNA (Sawaoka et al., 2003) possibly through a p38-dependent mechanism (Tchen et al., 2004). We studied the effects of aurothiomalate also on TTP mRNA expression. Aurothiomalate had no effect on IL-1β-induced TTP mRNA expression in H4 chondrocytes. However, the results do not rule out the possibility that aurothiomalate may alter the activation or activity of TTP.

Little is known on the pathophysiological and pharmacological factors that regulate COX-2 mRNA stability. Taxanes seem to increase COX-2 mRNA stability by a mechanism related to HuR (Subbaramaiah et al., 2003). Also transforming growth factor β (Sheng et al., 2000; Harding et al., 2006) and glycogen synthase kinase-3β (Thiel et al., 2006) have been reported to increase COX-2 mRNA stability whereas dexamethasone and thalidomide were found to destabilize COX-2 mRNA (Ristimäki et al., 1996; Lasa et al., 2001; Jin et al., 2007). In addition, protein kinase C-βII (Yu et al., 2003; Subbaramaiah et al., 2003) and JNK (Nieminen et al., 2006) pathways may be involved in the regulation of COX-2 mRNA stability. The present study adds aurothiomalate to the compounds that down-regulate COX-2 expression by destabilizing its mRNA.

Disease modifying anti-rheumatic drugs suppress inflammation, and reduce cartilage degradation and bone erosion in arthritis. The detailed molecular mechanism of action of many traditional disease modifying anti-rheumatic drugs is poorly understood. Their effects on leukocytes and synovial cells have been investigated but very little is known on their effects on chondrocytes in affected cartilage. In arthritis, COX-2 is highly expressed in the cartilage, and its expression is enhanced by pro-inflammatory cytokines IL-1 and TNF-α (Hardy et al., 2002; Abramson and Yazici 2006). Because chondrocytes seem to be a forgotten target in arthritis, we decided to investigate the effects of traditional disease modifying anti-rheumatic drugs on COX-2 expression and PGE$_2$ production in chondrocytes. Interestingly, aurothiomalate was effective whereas cyclosporin A, hydroxychloroquine, leflunomide, its active metabolite A771726, methotrexate and sulfasalazine had only little or no effect. Aurothiomalate has proved to be anti-inflammatory and anti-erosive in the treatment of arthritis, and it has been shown to control symptoms and disease activity of rheumatoid arthritis (Luukkainen et al., 1977; Munro et al., 1998; Sander et al., 1999, Lehman et al., 2005). However, its adverse effects have limited its use. The present results extend our understanding on the molecular mechanisms of action of aurothiomalate and they can be utilized in the drug development for arthritis.

Gold was originally introduced to the treatment of tuberculosis. Although ineffective in tuberculosis it was found serendipitously to have anti-rheumatic properties, and has been used to treat patients with rheumatoid arthritis since the 1920s (Forestier, 1935). Despite extensive clinical history in the treatment of rheumatoid arthritis, the mechanisms of anti-inflammatory action of gold compounds have not yet been fully defined. Aurothiomalate has been reported to suppress IL-8 production in endothelial cells, peripheral blood mononuclear cells (Seitz et al., 1992) and rheumatoid synoviocytes (Loetscher et al., 1994) as well as to inhibit IL-12 production and IL-2 receptor expression in T cells (Sfikakis et al., 1993). Seitz has reported inhibition of IL-1β production and caspase-1 activity in THP-1 monocytes (Seitz et al., 2003). Aurothiomalate has also been shown to inhibit serum IL-6 levels in rheumatoid arthritis patients (Lacki et al., 1995). Yanni reported that administration of aurothiomalate resulted in reduced accumulation of monocytes and macrophages in rheumatoid arthritis synovial membranes and significant inhibition of TNF-α, IL-1β and IL-6 expression in these cells (Yanni et al., 1994). The pharmacology of gold compounds in rheumatoid arthritis has been presented and discussed in an excellent review by Noriega and Harth (1997). However, very little is known about the molecular effects of aurothiomalate on chondrocytes although it has been shown to retard cartilage degradation (Luukkainen et al., 1977; Sander et al., 1999, Lehman et al., 2005). Recently, aurothiomalate was shown to inhibit iNOS expression and NO production in H4 chondrocytes and in human osteoarthritic cartilage (Vuolteenaho et al., 2005). In the present study, we found that aurothiomalate reduced PGE$_2$ production and COX-2 expression in chondrocyte cultures and in human cartilage and that was related to inhibition of COX-2 mRNA stability and to reduced HuR expression. Because HuR is a factor that regulates the stability of the mRNAs of COX-2 and many other inflammatory

genes, some of the above mentioned effects of aurothiomalate on inflammatory mediators may be explained by the same molecular mechanism, that we relate to reduced COX-2 expression, i.e. through suppressed HuR expression and enhanced degradation of the target mRNA.

In conclusion, the present results show that aurothiomalate reduces COX-2 expression and PGE_2 production in IL-1β-treated chondrocytes and in human cartilage. The mechanism of action is most likely related to aurothiomalate's ability to enhance COX-2 mRNA degradation. Aurothiomalate inhibited also the expression of a mRNA stability regulating protein, HuR, which provides a provisional mechanism for COX-2 mRNA destabilization. The results offer a novel mechanism for the anti-inflammatory action of aurothiomalate, and stress chondrocytes and their products as important targets in drug development for arthritis.

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