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Regulation of microsomal prostaglandin E_2 synthase-1 and 5-lipoxygenase-activating protein/5-lipoxygenase by 4-hydroxynonenal in human osteoarthritic chondrocytes

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Regulation of microsomal prostaglandin E_2 synthase-1 and 5-lipoxygenase-activating protein/5-lipoxygenase by 4-hydroxynonenal in human osteoarthritic chondrocytes

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RÉSUMÉ

L'arthrose (OA) est une maladie dégénérative et multifactorielle caractérisée par une destruction de cartilage, une formation d'ostéophytes et une inflammation au niveau de la membrane synoviale. Le 4-hydroxynonénal (HNE), un produit final de la peroxydation lipidique, a été identifié récemment comme un facteur catabolique et un médiateur inflammatoire dans le cartilage arthrosique humain. Notre projet vise à étudier l'effet du HNE sur la régulation de la prostaglandine E_2 synthase-1 microsomale (mPGES-1) et de la protéine activante 5-lipoxygénase (FLAP)/5-lipoxygénase (5-LOX) dans les chondrocytes arthrosiques humains. Lorsque les cellules sont traitées une seule fois avec 10 μM HNE, les résultats de Western blot et de PCR en temps réel montrent que l'expression de la cyclooxygénase-2 (COX-2) et de la mPGES-1 augmente de manière significative et atteint respectivement le maximum après 8 et 16 heures d'incubation puis diminue graduellement. Cependant, lorsque les cellules sont traitées plusieurs fois avec 10 μM HNE à 2 heures d'intervalle, l'expression de la COX-2 et de la mPGES-1 augmente en fonction du temps sans subir une baisse après 24 heures d'incubation. Le HNE induit l'activité du promoteur de la mPGES-1 via l'activation du facteur de transcription Egr-1. L'investigation de la $2^{ème}$ voie du métabolisme de l'acide arachidonique, à savoir 5-LOX/FLAP, montre que le HNE induit l'expression de FLAP après 24 heures de stimulation et celle de 5-LOX seulement après 48 heures. Ceci semble survenir à l'étape de transcription au cours de laquelle HNE induit l'expression de l'ARNm et l'activité du

promoteur du gène 5-LOX. Nous avons démontré aussi que le niveau de leukotriène B_4 (LTB_4) augmente et suit le même profil que celui de la 5-LOX. L'étude des mécanismes moléculaires susceptibles d'être impliqués dans la régulation de la 5-LOX/FLAP par le HNE montre que ce dernier stimule leur expression via l'action de prostaglandine E_2 (PGE_2) et du facteur de croissance transformant-beta 1 (TGF-β1).

En conclusion, notre étude démontre que le HNE induit à court-terme d'incubation la voie de COX-2/mPGES-1 puis par la suite stimule celle de FLAP/5-LOX à long-terme d'incubation dans les chondrocytes arthrosiques humains. Ces résultats suggèrent que la mPGES-1 et 5-LOX/FLAP sont des potentielles cibles thérapeutiques intéressantes pour contrôler la production de PGE_2 et LTB_4 dans OA.

Mots clés:

Arthrose, Inflammation, Chondrocytes, Peroxydation lipidique, 4-hydroxynonénal, Cyclooxygénase-2, Prostaglandine E_2 synthase-1 microsomale, Prostaglandine E_2, 5-lipoxygénase, Protéine activante 5-lipoxygénase, Leukotriène B_4.

SUMMARY

4-hydroxynonenal (HNE), a lipid peroxidation end-product, is produced abundantly in osteoarthritic (OA) articular tissues. Recently, we reported that HNE-induced cyclooxygenase-2 (COX-2) decreased gradually in human OA chondrocytes after 8 h of incubation. This study aimed to investigate whether COX-2 down-regulation is attributed to HNE depletion and is responsible for the switch from COX-2 to 5-lipoxygenase-activating protein (FLAP)/5-lipoxygenase (5-LOX). Treatment of chondrocytes with 10 μM HNE induced prostaglandin E_2 (PGE_2) release as well as COX-2 and microsomal prostaglandin E_2 synthase-1 (mPGES-1) expression at the protein and mRNA levels, with a plateau reached at 8-16 h of incubation, followed by a subsequent decline. However, 8 repeated treatments with 10 μM HNE prevented the reduction of COX-2 and mPGES-1 expression. We demonstrated that HNE induced mPGES-1 promoter activity mainly through transcription factor Egr-1 activation. On the other hand, when COX-2 expression decreased, leukotriene B_4 (LTB_4) level rose after a long period of stimulation (48 and 72 h). At the mRNA level, HNE induced FLAP and 5-LOX expression after 24 and 48 h of stimulation, respectively. The addition of a nonspecific COX-2 inhibitor (naproxen) to cultured chondrocytes revealed that FLAP and 5-LOX regulation by HNE required PGE_2 production. Furthermore, our data showed that 10 μM HNE significantly induced transforming growth factor-beta 1 (TGF-β1) production. The addition of anti-TGF-β antibody to culture medium reduced HNE-induced 5-LOX/FLAP expression by 40%, indicating the

involvement of a TGF-β1-dependent mechanism. Our data demonstrate that the shunt to the FLAP/5-LOX pathway in HNE-induced human OA chondrocytes is attributed to COX-2 inhibition, probably due to HNE depletion. PGE_2 and TGF-β1 are suggested to be involved in this regulation. Further experiments are in progress to determine other molecular mechanisms underlying this switch in OA chondrocytes.

Key words:

Osteoarthritis, Inflammation, Chondrocytes, Lipid peroxidation, 4-hydroxynonenal, Cyclooxygenase-2, Microsomal prostaglandin E_2 synthase-1, Prostaglandin E_2, 5-lipoxygenase, 5-lipoxygenase-activating protein, Leukotriene B_4.

TABLE OF CONTENTS

Résumé...iii

Summary...v

Table of contents...vii

List of figures...ix

List of abbreviations...x

Acknowledgments...xiii

CHAPTER I: INTRODUCTION...1

1. SYNOVIAL JOINTS AND ARTICULAR CARTILAGE...2

1.1 Structure...2

1.2 Composition...5

1.3 Metabolic and biochemical characteristics...6

2. OSTEOARTHRITIS (OA)...8

2.1 Definition and classification...8

2.2 Epidemiology of OA...9

2.2.1. Prevalence and incidence of OA...9

2.2.2 Risk factors for OA...10

3. PATHOPHYSIOLOGY OF OA...12

3.1 Matrix metalloproteinases (MMPs) and aggrecanases...13

3.2 Roles of cytokines in OA...14

3.3 Reactive oxygen species (ROS)...15

4. 4-HYDROXYNONENAL (HNE)……16

4.1 Synthesis and characteristics of HNE……..….16

4.2 Metabolism of HNE…….…….18

4.3 HNE binding proteins…….……..21

4.4 HNE and signaling pathways……21

5. ARACHIDONIC ACID METABOLISM……...22

5.1 Cyclooxygenase (COX)……..……..25

5.2 Prostaglandin E_2 synthase (PGES).……...27

5.3 Prostaglandin E_2 (PGE_2)…….. 28

5.4 5-lipoxygenase (5-LOX).……..29

5.5 Regulation of 5-LOX expression……..29

5.6 5-LOX-activating protein (FLAP)……31

6. OBJECTIVES AND HYPOTHESIS……..…….33

CHAPTER II: MATERIALS AND METHODS……....……34

CHAPTER III: RESULTS…….……...41

CHAPTER IV: DISCUSSION……60

CHAPTER V: CONCLUSION…….…….72

CHAPTER VI: REFERENCES…….……...75

LIST OF FIGURES

Figure 1: Structure of synovial joint ……..……..3

Figure 2: Structure of articular cartilage in adults ……..4

Figure 3: Imbalance between anabolic factors (anti-inflammatory) and catabolic factors (pro-inflammatory), leading to the destruction of cartilage...…..7

Figure 4: Structure of HNE with an α-β double bond and a hydroxyl group……17

Figure 5: Metabolism of HNE….……...……...20

Figure 6: Role of HNE in the development of OA…….……..…….23

Figure 7: Arachidonic acid metabolism…….24

Figure 8: COX-2 expression in the absence or presence of stimulations……..26

Figure 9: Intracellular organization of the critical enzymes involved in leukotriene biosynthesis……..32

Figure 10: HNE-induced PGE_2 production and COX-2 expression in human OA chondrocytes……..…43

Figure 11: HNE-induced mPGES-1 protein and mRNA expression in human OA chondrocytes……...…...46

Figure 12: HNE-activated mPGES-1 promoter…….48

Figure 13: HNE-induced LTB_4 biosynthesis and 5-LOX and FLAP mRNA expression in human OA chondrocytes……...…51

Figure 14: HNE-activated 5-LOX promoter in human OA chondrocytes……53

Figure 15: HNE-induced FLAP/5-LOX expression is PGE_2-dependent in human OA chondrocytes……. 55

Figure 16: HNE-induced FLAP/5-LOX expression is TGF-β1-dependent in human OA chondrocytes……..58

LIST OF ABBREVIATIONS

5-LOX: 5-lipoxygenase

AA: Arachidonic acid

ADAMTS: A disintegrin and metalloprotease with thrombospondin motifs

Col: Collagen

Col II: Type II collagen

Col V: Type V collagen

COX: Cyclooxygenase

COX-2: Cyclooxygenase-2

cPGES: Cytosolic prostaglandin E_2 synthase

$cPLA_2$: Cytosolic phospholipase A_2

CYS: Cysteine

cys-LTs: Cysteinyl-leukotrienes

DHN: Dihydroxy-2-nonene alcohol

ECM: Extracellular matrix

Egr-1: Early growth response protein-1

EIA: Enzyme immunoassay

FLAP: 5-lipoxygenase-activating protein

GM-CSF: Granulocyte/macrophage colony-stimulating factor

GSH: Glutathione

GST: Glutathione-S-transferase

HIS: Histidine

HNA: 4-hydroxy-2-nonenoic acid

HNE: 4-hydroxynonenal

IL-1β: Interleukin-1 beta

iNOS: Inducible nitric oxide synthase

JNK: c-Jun N-terminal kinases

LOX: Lipoxygenase

LPO: Lipid peroxidation

LPS: Lipopolysaccharides

LTB_4: Leukotriene B_4

LTs: Leukotrienes

LYS: Lysine

MAPK: Mitogen-activated protein kinases

MDA: Malondialdehyde

MMPs: Matrix metalloproteinases

mPGES-1: Microsomal prostaglandin E_2 synthase-1

NF-κB: Nuclear factor-kappa B

NO: Nitric oxide

O_2^-: Superoxide anion

OA: Osteoarthritis

PG: Proteoglycan

PGE_2: Prostaglandin E_2

PGES: Prostaglandin E_2 synthase

PGH_2: Prostaglandin H_2

PPARγ: Peroxisome proliferator-activated receptor gamma

RA: Rheumatoid arthritis

ROS: Reactive oxygen species

TGF-β1: Transforming growth factor-beta 1

TIMP: Tissue inhibitor of metalloproteinases

TNF-α: Tumor necrosis factor alpha

VDR: Vitamin D receptors

VDRE: Vitamin D receptor element

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CHAPTER I: INTRODUCTION

1. SYNOVIAL JOINTS AND ARTICULAR CARTILAGE

Synovial joints are structures consisting of bone, hyaline cartilage, synovial membrane and joint capsule filled with synovial fluid **(Fig. 1)**. Synovial fluid serves as a protective barrier by preventing contact between surfaces and provides nutrients to the hyaline cartilage (Schmidt et al., 2007). The latter covers the surface of bone and provides low-friction gliding surface with strong compressive strength and resistance; therefore it facilitates the movement of bones without difficulties and pain. Destruction of cartilage brings extreme pain during the movement, which we often see in osteoarthritis (OA) patients. In this section, we will discuss the structure, composition and biochemical characteristics of cartilage.

1.1 Structure

Morphologically, the hyaline cartilage is a connective tissue, which divides into four layers from the exterior to interior: superficial zone, transitional (middle zone), deep zone and calcified zone. The thickness of different layers depends on the inside component and the intensity of force applied (Poole et al., 2001).

The superficial zone is the thinnest layer consisting of flattened cells aligned parallel to the surface. It is covered by the protein lubricin and synovial fluid. This parallel arrangement gives the greatest tensile strength to cartilage **(Fig. 2)**. The transitional zone of extracellular matrix (ECM) is formed by spheroid shaped cells with lower cell density and high concentration of

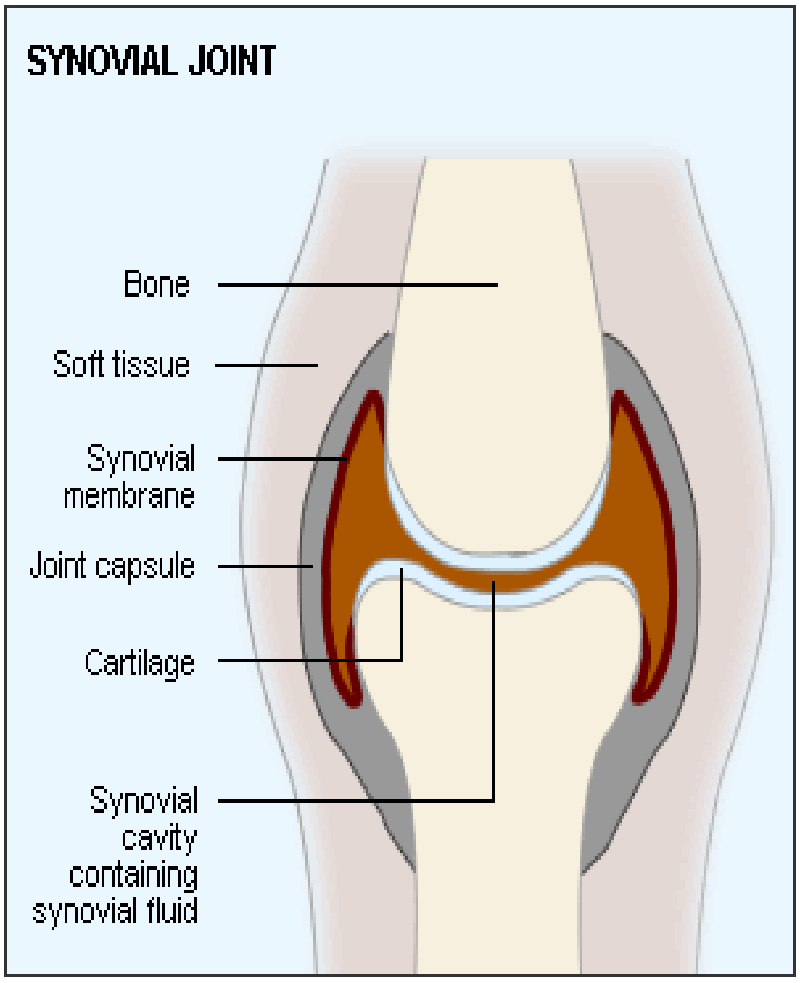

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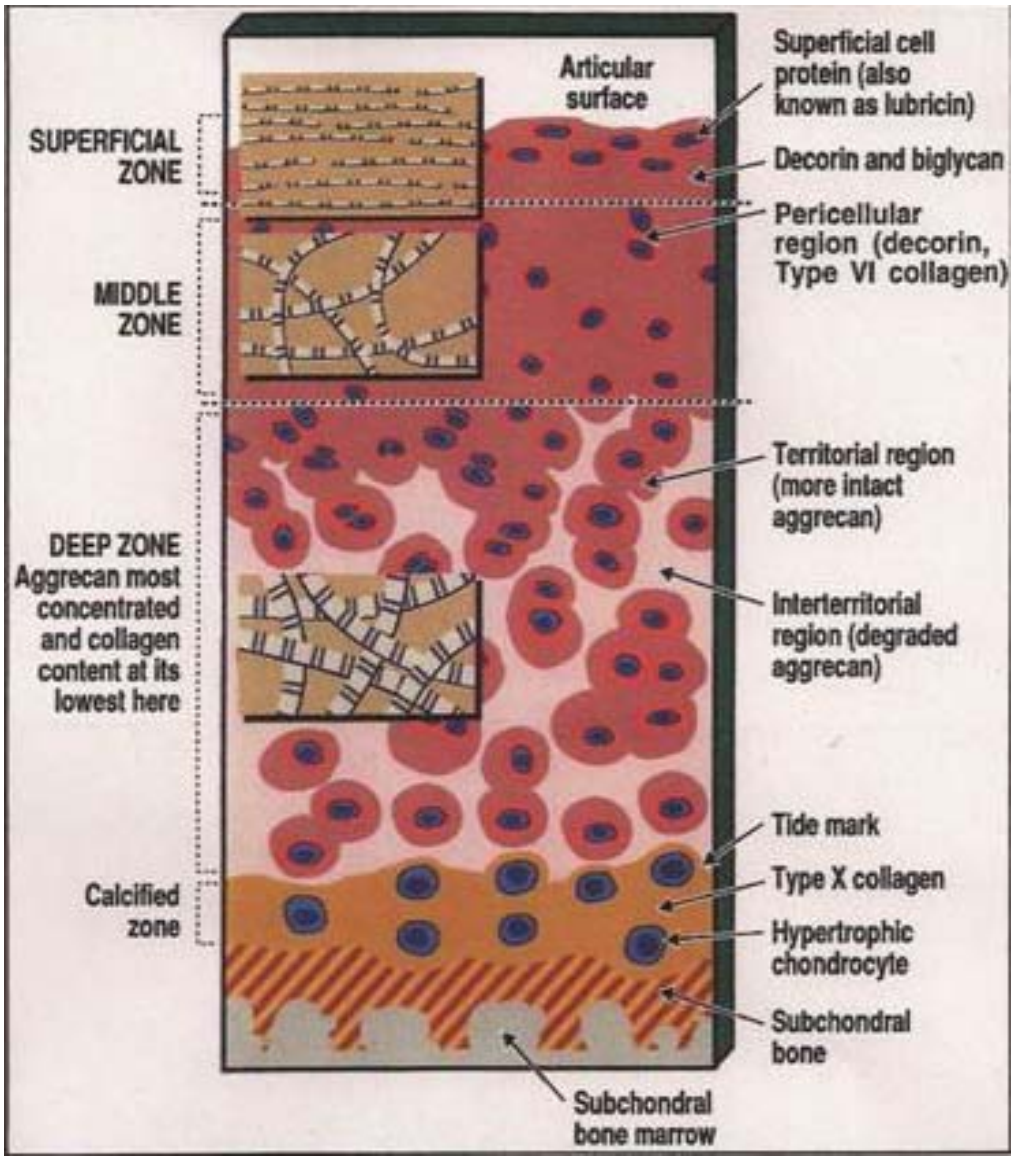
Figure 1: Structure of synovial joint



(Poole et al., 2001)

Figure 2: Structure of articular cartilage in adults

macromolecules, such as proteoglycans and collagens (Bhosale and Richardson, 2008).

Chondrocytes present in the deep zone have spheroid shape with perpendicular arrangement. Highest level of proteoglycan is observed in this zone. The calcified zone, the most profound zone of hyaline cartilage, contains low quantity of cells. However, the chondrocytes in this zone are special because they show hypertrophic phenotype and have capacity of synthesizing type V collagen (Col V) **(Fig. 2)**. This feature provides an important structural integrity to the less elastic subchondral bone (Aigner and McKenna, 2002).

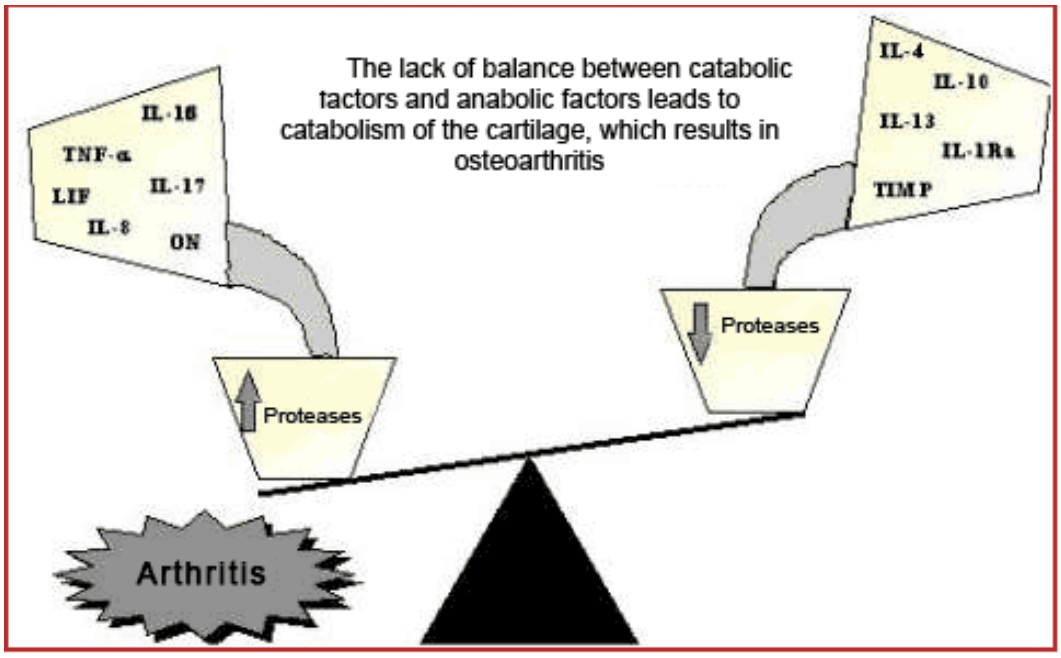
1.2 Composition

The hyaline cartilage is an avascular and alymphatic ECM composed of only chondrocytes. This ECM consists of 70% of water and 30% of collagen (Col), proteoglycan (PG) and elastic fibers. There exists many types of collagen including type II collagen (Col II), Col VI, Col IX, Col X and Col XI; among them Col II is the most abundantly present in ECM. These components help to maintain the tension and the movement of articulations as well as to provide essential nutrients for chondrocytes (Gardner, 1983;Bhosale and Richardson, 2008).

1.3 Metabolic and biochemical characteristics

The characteristics of cartilage are determined by the structure and functions of molecules presenting in the ECM. Chondrocytes existing in the cartilage are very active and able to secrete various molecules such as cytokines, growth factors and enzymes. Interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) produced abundantly by chondrocytes can generate different cellular responses through various signal pathways. For example, IL-1β stimulates inducible nitric oxide synthase (iNOS) expression through p38 Mitogen-activated protein kinases (MAPK) pathway (Badger et al., 1998). It can also stimulate nuclear factor-kappa B (NF-κB) that translocates to the nucleus to activate various genes. Furthermore, cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) synthesis were determined to be upregulated by IL-1β and TNF-α in explants of human OA knee menisci (LeGrand et al., 2001).

As ECM is an avascular and alymphatic tissue, the transport of nutrients to chondrocytes is through diffusion. In normal cartilage, degradation (catabolism) and repair (anabolism) are in equilibrium, which is controlled by the synthesis of ECM components such as collagen, matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMP). In OA, catabolism is more active than anabolism. The overproduction of MMPs extensively degrades cartilage components, which cannot be repaired by anabolism mediator TIMP and results in OA **(Fig. 3)**.



(csbioactive website, osteoarthritis)

Figure 3: imbalance between anabolic factors (anti-inflammatory) and catabolic factors (inflammatory), leading to the destruction of cartilage.

2. OSTEOARTHRITIS (OA)

2.1 Definition and classification

OA is the most common form of arthritis and one of the most pervasive diseases in the Western society. According to the Arthritis Society, approximately 3 million Canadians suffer from OA. OA is a disease that affects a subset of the joints of the body, particularly the hips, knees and distal interphalangeal joints of the hands (Oliveria et al., 1995;Dieppe et al., 2002). Examination of an OA joint by arthroscopy or open surgery confirms a dramatic loss of articular cartilage (Gardner, 1983;Radin et al., 1991;Kerin et al., 2002). Histological examination of the cartilage confirms the gross anatomy and reveals a striking loss of ECM components (Poole, 1999;Felson et al., 2000). Depending on the region being examined, cartilage may be devoid of chondrocytes, presumably as a result of cell death, or contains clusters of chondrocytes that have undergone division, possibly in response to ECM depletion (Sandell and Aigner, 2001). Some chondrocytes appear to be synthesizing ECM at an elevated rate, as if attempting repair, while the synthetic rates of other chondrocytes are depressed (Haq et al., 2003). It is generally thought that an elevated ECM synthesis is a feature of early OA, while reduced synthetic rates occur later in the disease. Loss of articular cartilage is a progressive condition that develops in response to a variety of stimuli: mechanical or environmental, and is orchestrated by growth factors and cytokines acting through several signaling cascades (Radin et al., 1991;Pelletier et al., 1993;Arend and Dayer, 1995;Westacott and Sharif, 1996;Kerin et al., 2002). The cartilage

degradation is characterized by the loss of ECM components and qualitative changes of collagen. The precise mechanisms responsible for the initiation of cartilage degradation remain to be clarified.

OA is classified into two groups: primary (idiopathic) and secondary. Primary OA is the most common form and has no known cause, although it is often related to aging and heredity. Secondary OA is related to antecedent factors including trauma, congenital or developmental diseases, metabolic diseases, endocrine diseases and other bone diseases.

2.2 Epidemiology of OA

2.2.1 Prevalence and incidence of OA

OA is an extremely common joint disorder in the whole population. Its high prevalence and the frequency of OA-related physical disability make OA, especially in the elderly, one of the leading causes of disability. OA of the hip and knee represent two of the most significant causes of adult pain and physical disability. OA has a higher prevalence, and more often exhibits a generalized distribution in women than in men. Before the age of 50, men have a higher prevalence than women, but after the age of 50, women have a higher prevalence. This sex difference in prevalence further increases with age (Felson and Zhang, 1998;Nevitt et al., 2001). Overall, OA occurs frequently in knees, hands, hips,

back, neck, wrists and ankles. Many people have joint symptoms without X-ray change and vice versa.

2.2.2 Risk factors for OA

Risk factors for OA include systemic and local biomechanical factors

Systemic factors:

Age, sex and ethnicity: the most potent systemic vulnerabilities are an increasing age and the female gender. Disease incidence and prevalence increase dramatically with age. The Framingham study found that 27% of those aged 63 to 70 had radiographic evidence of knee OA, increasing to 44% in the over 80 age group (Felson et al., 1995). Racial factor is another systemic factor with Asian people having very low rates of hip OA (Haq et al., 2003).

Genetics: OA is a group of clinically heterogeneous disorders. Many genes have been linked to OA. There is most concordance with chromosomes 2q, 4 and 16. Families have been found with rare autosomal dominant patterns of inheritance of OA. The defective genes are often coding for structural proteins of the ECM of the joint and collagen (Haq et al., 2003;Gabay et al., 2008).

Hormonal status: some women after age 50 develop “menopausal arthritis” at the time of menopause. These gender- and age-related prevalence patterns are consistent with a role for post-menopausal hormone deficiency in increasing the risk of OA. Estrogen loss has been strongly implicated as a risk factor.

Epidemiologic studies provide evidence that estrogen replacement therapy is associated with a reduction in the risk of knee and hip OA (Nevitt et al., 2001).

Nutritional factors: people with lower vitamin C and vitamin D levels in blood have a threefold risk of progression of knee OA (Hunter et al., 2002). Vitamin C protects against damage by reactive oxygen species (ROS) and serves as a cofactor for enzymes contributing to Col II synthesis. Low dietary vitamin D intake increases the risk of knee OA progression (Bergink et al., 2009).

Local biomechanical factors

Obesity: many recent studies allow us to better understand the relationships between OA and obesity (Gabay et al., 2008). Although it is evident that mechanical components contribute to joint destruction in overweight people, OA is considered not only a disease of articular cartilage but also a systemic disorder in which circulating factors linked to altered lipid and glucose metabolism may explain the diversity of pathophysiological changes found in generalised OA. However, the potential contribution of adipose-derived cytokines in OA would not preclude the involvement of other mechanisms, including activation of mechanoreceptors and vascular dysfunction in the subchondral bone.

Major joint injury: with a major joint injury, a person can sustain permanent damage of many of the structures within a joint. This damage alters its biomechanics, increases stress across particular areas of the joint and often

dramatically increases the risk of OA. The Framingham study found men with a history of knee injuries had a relative risk of 3.5 for subsequent knee OA; for women the relative risk was 2.2 (Felson, 1990).

Occupational and athletic activities: OA is common in those performing heavy physical works, especially if this involves knees bending, squatting, or kneeling. Dockers and miners have been found to have a higher prevalence of knee OA than those in sedentary jobs (Partridge and Duthie, 1968). Epidemiologic study has demonstrated that participation in certain competitive sports increases the risk for OA (Buckwalter and Lane, 1997b). Sports activities that appear to increase the risk for OA include those that demand high-intensity, acute, direct joint impact as a result of contact with other participants, playing surfaces, or equipment (Buckwalter and Lane, 1997a). Repetitive joint impact and torsional loading also appear to be associated with joint degeneration.

3. PATHOPHYSIOLOGY OF OA

OA is one of the most frequent health problems for older people. It is a multifactorial degenerative joint disease characterized by the destruction of articular cartilage and synovial inflammation. The principal factors can be sex, gene, strenuous exercise, excess weight and injury (Hunter et al., 2002;Ouedraogo et al., 2008). The pathophysiology of OA is not completely understood, but its progression is being evaluated. The development of OA is usually separated into

three stages. Stage I is the imbalance between anabolic and catabolic metabolism, in which excessively synthesized proteases damage the cartilage matrix **(Fig. 3)**. During stage II, the erosion of cartilage surface and inflammation of synovial membrane are observed, and the breakdown products are released into the synovial fluid. In stage III, these products are ingested by the synovial cells through phagocytosis, which is accompanied by the production of protease and proinflammatory cytokines (Martel-Pelletier, 2004). OA does not only cause the deterioration of cartilage but also the synovial membrane where an inflammatory reaction is often present. In this section, we will discuss the factors mediating in the development of OA, such as MMPs, aggrecanase protease, cytokines and ROS.

3.1 Matrix metalloproteinases (MMPs) and aggrecanases

MMPs are considered to be the main proteases responsible for proteolytic degradation of collagen and aggrecan in cartilage ECM (Martel-Pelletier et al., 2001). The expression of several MMPs was elevated in synovial tissues of OA patients. Degradation of Col II in the matrix is an irreversible step in OA and it was thought to be catalyzed by the MMP-1. Newer evidences showed that MMP-13 also has this effect and is the most reactive MMP in the degradation of OA cartilage. It targets Col II five times more rapidly than MMP-1 (Reboul et al., 1996). More members of MMPs (MMP-3, MMP-8, MMP-14) were identified. MMP-3 and MMP-14 degrade proteoglycan and are involved in the activation of other pro-MMPs. All of these MMPs are able to degrade different types of

collagen with different extents and mechanisms (Moilanen et al., 2003). Several aggrecanases have been identified: A disintegrin and metalloprotease with thrombospondin motifs (ADAMTS)-1, ADAMTS-4 and ADAMTS-5. They are members of the ADAMTS family of aggrecanases. They degrade aggrecans during cartilage destruction (Nagase and Kashiwagi, 2003).

3.2 Roles of cytokines in OA

Proinflammatory cytokines produced during synovial membrane inflammation upregulate MMPs gene expression and promote further progression of cartilage lesions in OA. They are synthesized by activated synoviocytes and by articular cartilage itself (Fernandes et al., 2002). Enhanced amounts of these mediators are found in OA synovial tissues compared to normal tissues. Among the cytokines, IL-1β and TNF-α are considered to be the principal mediators of joint destruction. It is evident that, in animal model experiments, inhibiting IL-1β synthesis and activity prevent cartilage damage, whereas blocking TNF-α reduces inflammation. This suggests that TNF-α drives inflammatory process and that IL-1β is more responsible for cartilage destruction (Martel-Pelletier, 2004). The main action of IL-1β is to suppress proteoglycan and collagen synthesis in parallel with the release of destructive protease in chondrocytes. Other cytokines involved in the development of OA include IL-8, IL-17 and IL-6. It has been showed that IL-6 deficient mice have a reduced joint inflammation (Van de Loo et al., 1997). These cytokines increase the number of inflammatory cells by upregulating other

proinflammatory cytokines, enhance the production of MMPs and stimulate the production of oxidative and inflammatory products synthesized by 5-lipoxygenase (5-LOX) (Guerne et al., 1989;Schroder, 1989). Use of specific anti-cytokine was found to reduce cartilage destruction in animal model; therefore, it was thought to be a treatment in the development of OA.

Several anti-inflammatory cytokines (IL-4, IL-10 and IL-13) were also found at an increased level in synovial tissues. They have a protective role from the degradation of cartilage. They decrease the production of IL-1β, TNF-α and MMPs, upregulate TIMP-1 and inhibit PGE_2 release (Hart et al., 1989;Jovanovic et al., 1998). Since IL-4 exerts its effect at the translational level and IL-10 at the transcriptional level, synergic protection was observed when these two cytokines are given as a combination therapy (Joosten et al., 1997). In this context, modulation of cytokines that control the gene expression of MMPs becomes the target for drug development in the treatment of OA.

3.3 Reactive oxygen species (ROS)

ROS are produced by chondrocytes and synoviocytes in response to mechanical stress and inflammatory mediators. Recently, ROS were found to be involved in the degradation of cartilage (Henrotin et al., 2003). The main ROS produced in OA are nitric oxide (NO) and superoxide anion (O_2^-). NO is synthesized mainly by iNOS enzyme. The production of NO is stimulated by IL-

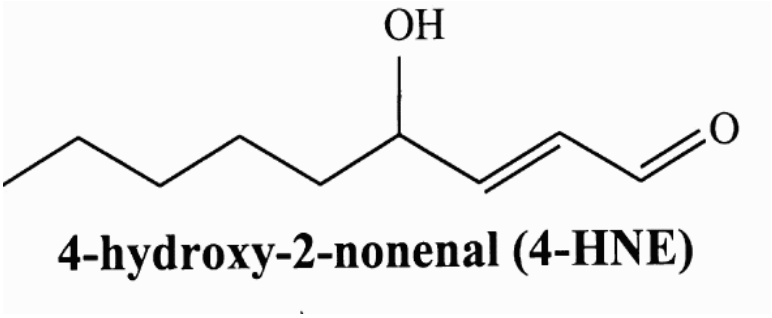
1β, TNF-α and lipopolysaccharides (LPS) and is inhibited by anti-inflammatory cytokines such as IL-4 and IL-10 (Stadler et al., 1991). In OA, an insufficient antioxidant capacity is not able to detoxify ROS; therefore oxidative stress will take place. Increased ROS induces chondrocyte apoptosis and cartilage matrix breakdown by upregulating the expression of MMPs (MMP-3 and MMP-13). It also modulates the production of pro-inflammatory mediators. However, some evidence had demonstrated that ROS are produced at a low level in chondrocytes and may contribute to the maintenance of cartilage homeostasis. It was observed that a decrease in levels of ROS reduces the ability of articular chondrocytes to regulate pH (Milner et al., 2007;Gibson et al., 2009).

4. 4-HYDROXYNONENAL (HNE)

4.1 Synthesis and characteristics of HNE

Oxidative stress initiates many signaling cascades of cellular functions including inflammation, cell proliferation and chemoattraction. Under oxidative stress, lipid peroxidation (LPO) of membrane polyunsaturated fatty acids proceeds through a free radical chain reaction to yield lipid peroxides. They are subsequently decomposed and generate several end products such as aldehydes.

HNE was identified to be the most produced and cytotoxic aldehyde during lipid peroxidation. Due to its long half-life and α-β double bond, HNE can react easily with nucleic acids, proteins and phospholipids **(Fig. 4)**. It also functions as a



(Pappa et al., 2003)

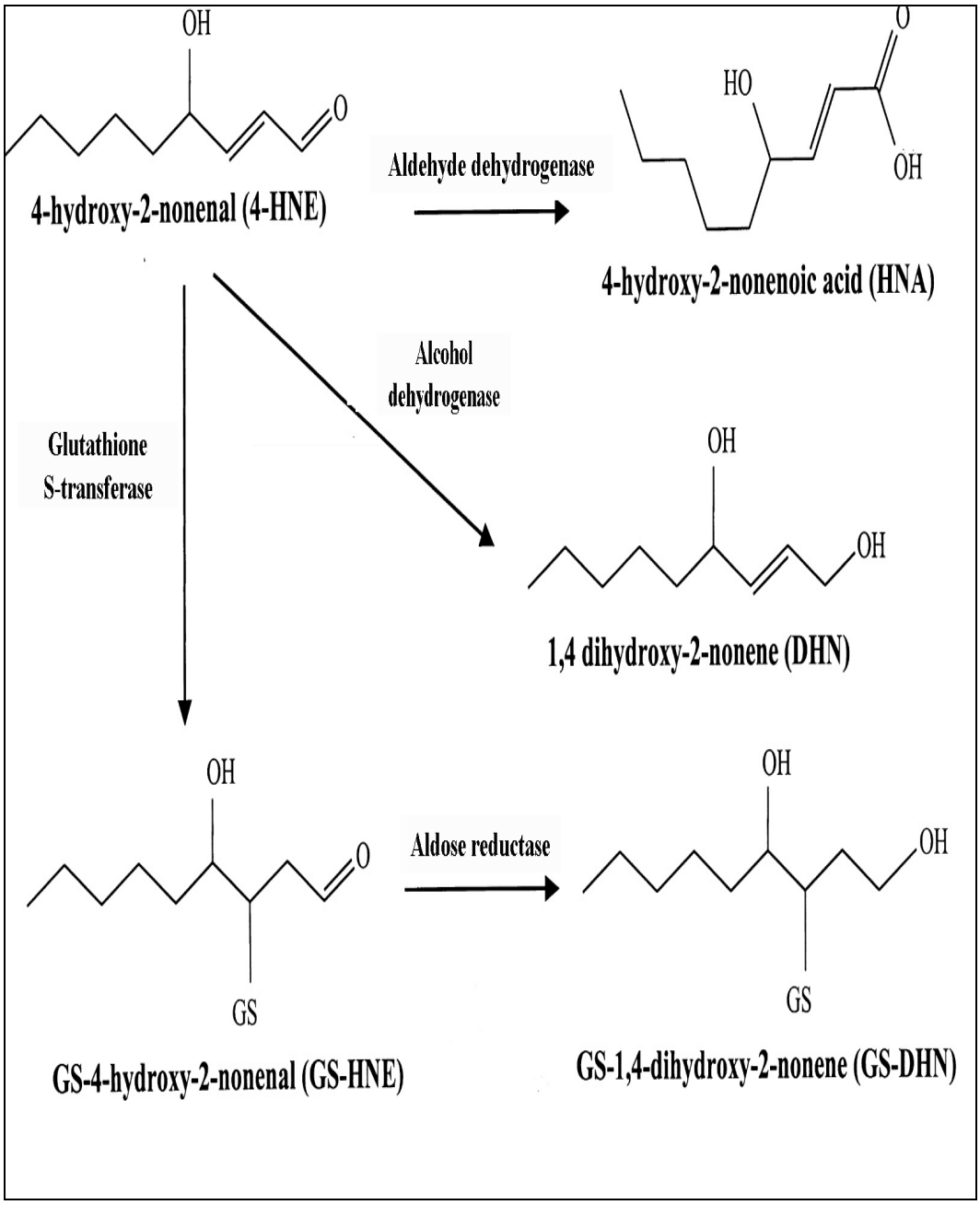
Figure 4: Structure of HNE with an α-β double bond and a hydroxyl group

bioactive molecule that affects numerous cell functions, including chemotactic activity, signaling transduction and gene expression (Esterbauer et al., 1991;Catala, 2009). Increased level of HNE was found in plasma, various organs and cells under oxidative stress conditions and in many rheumatological diseases, such as liver damage and chronic lymphedema (Comporti, 1985;Poli et al., 1987;Siems et al., 2002). Therefore, HNE Michael adduct can serve as a biomarker for the presence of oxidative stress and tissue damage (Zarkovic, 2003). A previous study also demonstrated higher HNE concentration in synovial fluids and in chondrocytes of OA patients compared to normal subjects (Morquette et al., 2006). The topic of HNE recently became controversial because HNE was found to be a normal constituent of membrane in mammals, at low concentration, without toxicity.

4.2 Metabolism of HNE

A low level of HNE is normally present in mammalian cells. Obviously, organisms have several enzymatic pathways to detoxify HNE in order to protect proteins and lipids from damage by aldehydic products. The best characterized HNE-metabolizing enzymes include the glutathione-S-transferase (GST), aldose reductase, aldehyde dehydrogenase and alcohol dehydrogenase **(Fig. 5)** (Pappa et al., 2003). HNE can be modified to non-toxic products through the following reactions: 1) Michael addition of glutathione (GSH) to HNE to produce GS-HNE, 2) the oxidation of HNE to 4-hydroxy-2-nonenoic acid (HNA), 3) the reduction to

unreactive 1,4-dihydroxy-2-nonene alcohol (DHN), 4) the interaction with carnosine, a dipeptide (beta-alanyl-L-histidine) present in high concentration in skeletal muscle (Aldini et al., 2002). Carnosine has numerous antioxidant properties and is able to scavenge ROS and unsaturated aldehydes formed during oxidative stress (Quinn et al., 1992;Babizhayev et al., 1994). Even with the rapid HNE metabolism, HNE-caused protein modification can occur. In different mammalian cells including hepatocytes, tumor cells and synovial fibroblasts, HNE metabolism is limited and a high level of protein-HNE adducts was observed after the addition of HNE (Siems and Grune, 2003). These HNE-modified proteins are believed to be involved in many impaired cells-related diseases. In this context, the degradation of intracellular HNE becomes one important part of the antioxidative defense systems.



(Modified from Pappa et al., 2003)

Figure 5: Metabolism of HNE

4.3 HNE binding proteins

HNE has a hydroxyl group and an α-β double bond, which make it highly reactive with specific amino acids of some proteins by the reaction of Michael addition to their particular functional groups. They were identified to be sulfhydryl group of cysteine, imidazole group of histidine and ε–amino group of lysine (Uchida and Stadtman, 1992a;Uchida and Stadtman, 1992b;Szweda et al., 1993). HNE can interact with many different cell proteins because it is quite stable and can pass through subcellular compartments. Several HNE-binding proteins were found, including glutamate transporter, proteasome and glyceraldehyde-3-phosphate dehydrogenase (Blanc et al., 1998;Okada et al., 1999;Tsuchiya et al., 2005). Due to its property of damaging DNA and proteins in cellular compartments, HNE was thought to be responsible for various pathophysiological effects associated with oxidative stress.

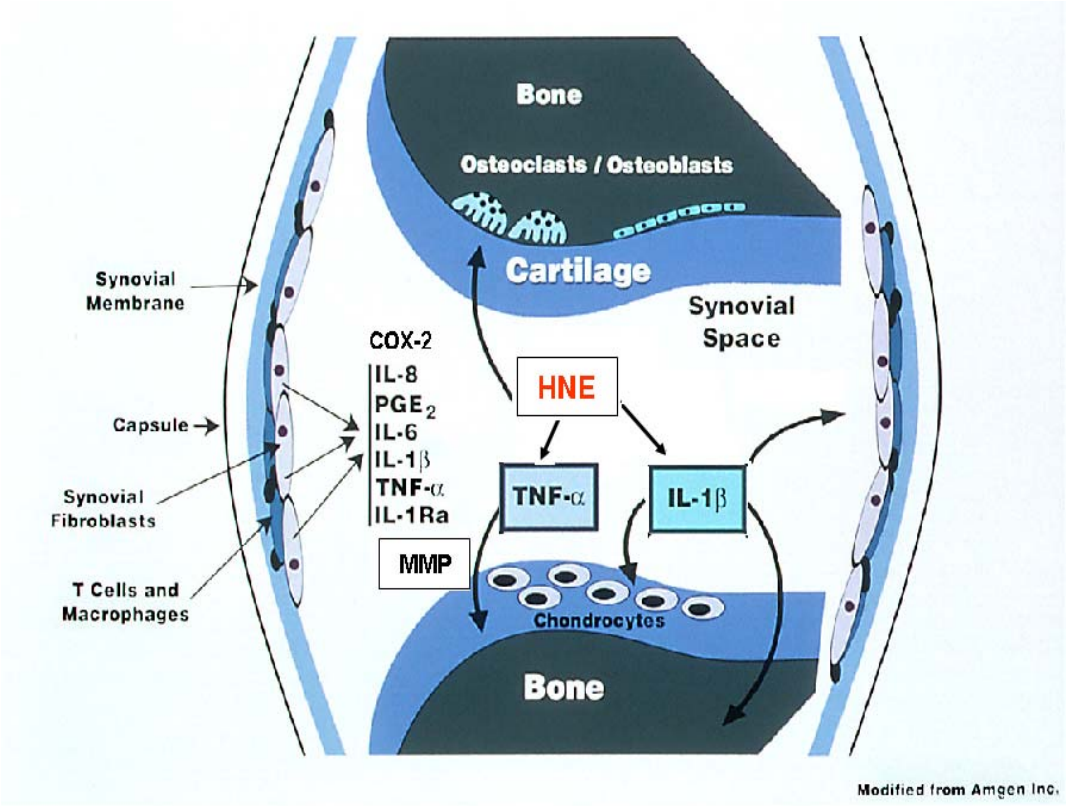
4.4 HNE and signaling pathways

Many studies suggested that HNE at low concentrations could be considered as a modulator of pathophysiological events by activating various signaling mechanisms, such as mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinases (JNK) and caspase pathways. Apoptosis is one example of intracellular responses under oxidative stress in which caspases cascade is activated by HNE (Liu et al., 2000;Vaillancourt et al., 2008). Interestingly, increasing evidence showed that HNE stimulates the production of MMPs,

including MMP-2, MMP-9, and MMP-13 through ERK and p38 MAPK pathway in various cell types (Seo et al., 2010;Lee et al., 2010). HNE also induces COX-2 expression through p38 MAPK **(Fig. 6)** (Kumagai et al., 2004). The roles of MMPs and inflammatory mediators in cartilage destruction have been extensively studied and defined. These findings suggest that HNE may be involved in the pathophysiology of OA in association with inflammatory responses.

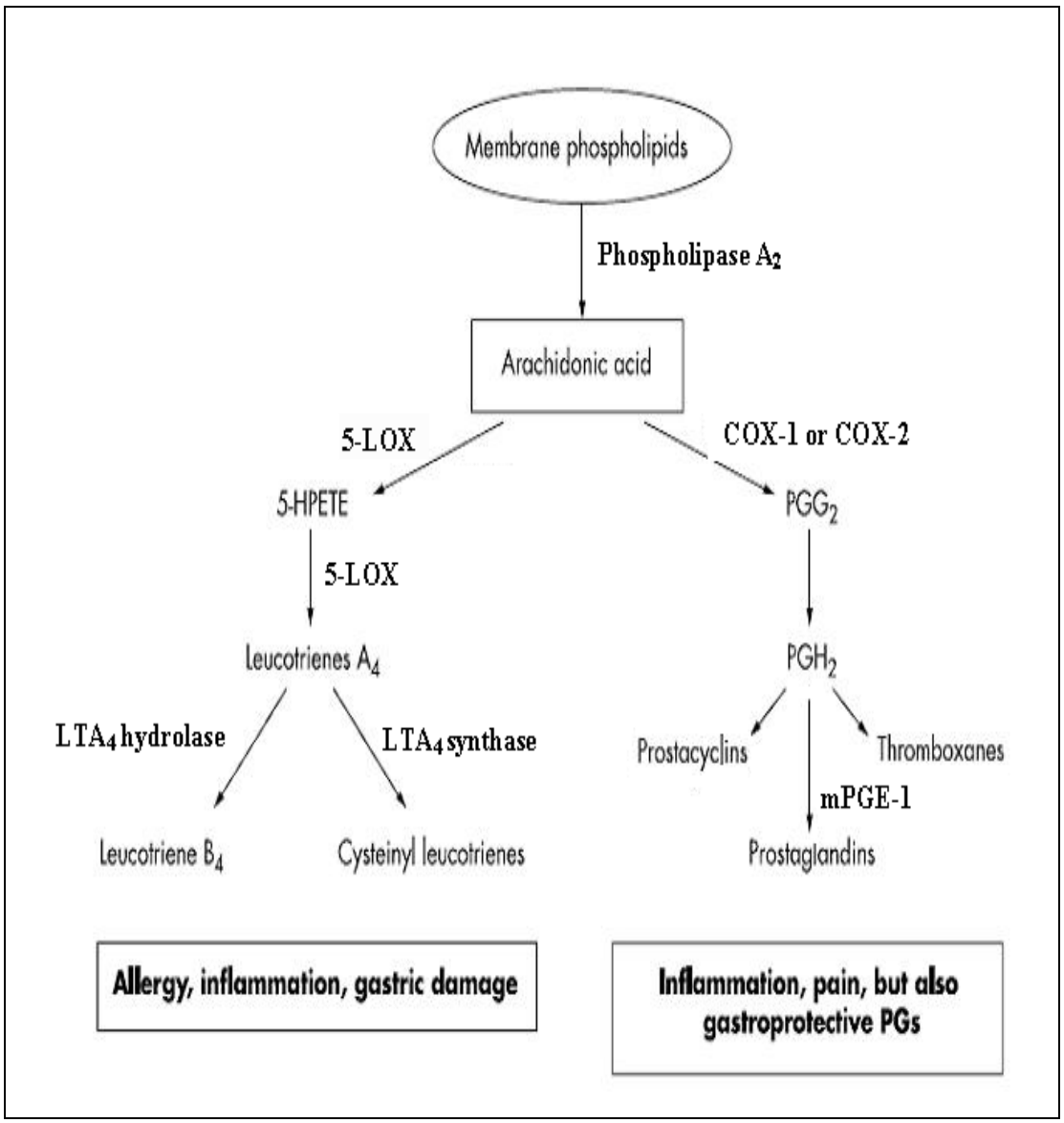
5. ARACHIDONIC ACID METABOLISM

During its metabolism, arachidonic acid (AA) is released from hydrolysis of cell membrane phospholipids by cytosolic phospholipase A_2 enzyme ($cPLA_2$). AA is then oxygenated to form PGG_2 by COXs, which subsequently reduce PGG_2 to the unstable intermediate called prostaglandin H_2 (PGH_2). PGH_2 quickly transform to prostacyclins, thromboxanes and prostaglandins by prostaglandin E_2 synthase (PGES) (Smith et al., 2000;Park et al., 2006). Another AA metabolism pathway is catalyzed by 5-lipoxygenase-activating protein (FLAP)/5-lipoxygenase (5-LOX), which are the pivotal enzymes to yield leukotrienes (LTs) **(Fig. 7)**. The amount of eicosanoid production is dependent on the release of AA by $cPLA_2$ (Park et al., 2006). The production and the regulation of these enzymes are discussed in detail in the following sections.



(Modified from Amgen Inc.)

Figure 6: Role of HNE in the development of OA.



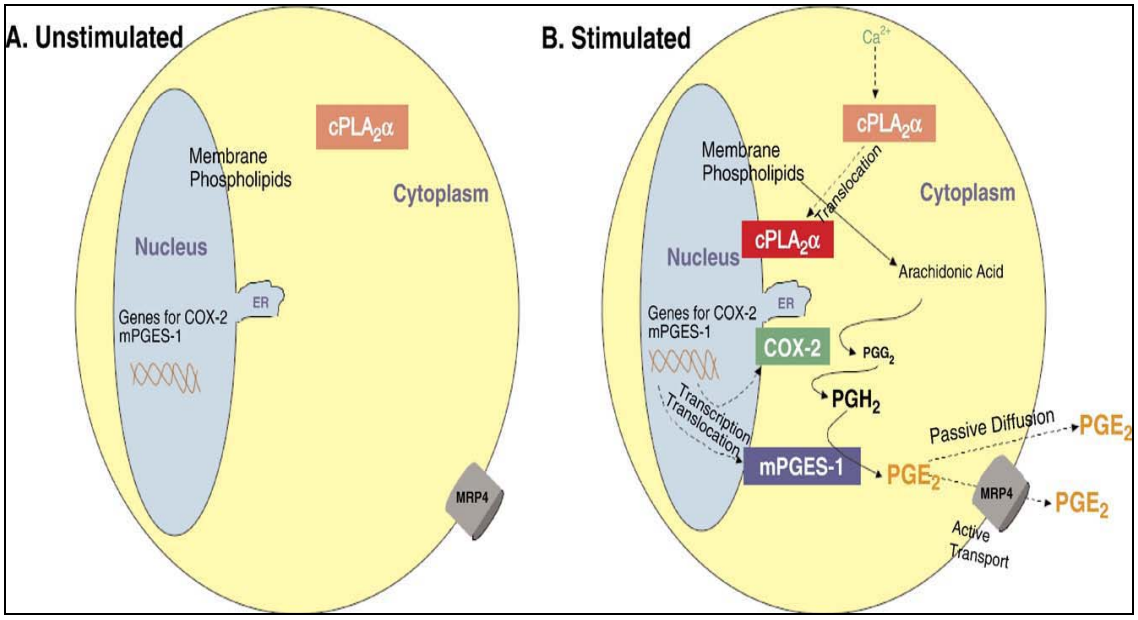
(Modified from Martel-Pelletier et al., 2003)

Figure 7: Arachidonic acid metabolism

5.1 Cyclooxygenase (COX)

Two COX isoforms have been identified: COX-1 and COX-2. They are encoded by different genes and expressed in different manners. COX-1 is a membrane-bound heme-containing glycoprotein expressed ubiquitously under normal conditions, whereas COX-2 is inducible and expressed by various stimuli (Martel-Pelletier et al., 2003;Uchida, 2008). Stimulation induces influx of calcium into the cell, leading to the translocation of $cPLA_2$ from the cytosol to the nuclear membrane where it enzymatically hydrolyzes membrane phospholipids to liberate AA **(Fig. 8)** (Park et al., 2006). Different stimuli that can induce COX-2 expression include pro-inflammatory IL-1β, TNF-α and growth factors, which promote prostaglandins synthesis and lead to further inflammation and carcinogenesis (FitzGerald, 2003). HNE was found to stimulate the expression of COX-2 and PGE_2 production in chondrocytes, suggesting it may induce OA development through inflammatory processes (Vaillancourt et al., 2007).

In human rheumatoid arthritis (RA) synovial membranes, immunostaining with anti-COX-2 antisera revealed the presence of COX-2 in infiltrating mononuclear cells, endothelial cells of blood vessels, and subsynovial fibroblast-like cells. IL-1β and phorbol myristate acetate enhance *de novo* mRNA and protein synthesis of COX-2 in RA synovial membrane and cultured synovial fibroblasts.



(Park et al., 2006)

Figure 8: COX-2 expression in the absence or presence of stimulations

COX-2 is also expressed in cartilage specimens from OA patients (Amin et al., 1997;Siegle et al., 1998). Osteoarthritic cartilage spontaneously releases PGE_2 in levels at least 50-fold higher than the normal cartilage and 18-fold higher than normal cartilage stimulated with cytokines and endotoxin. The production of PGE_2 was sensitive to the translation inhibitor Cycloheximide, indicating the inducible aspect of this mediator. These observations indicate that COX-2 expression is upregulated in arthritic synovial membranes and cartilage, and suggest that selective inhibition of COX-2 may result in the amelioration of arthritic diseases.

5.2 Prostaglandin E_2 synthase (PGES)

The terminal enzyme found in the COX pathway is called PGES, which collaborates with COX in the synthesis of prostaglandins from PGH_2. Three isoforms of PGES have been determined: cytosolic PGES (cPGES), microsomal PGES-1 (mPGES-1) and mPGES-2. cPGES is preferentially coupled with COX-1 to immediately produce PGE_2, whereas mPGES-1 is inducible as COX-2, both work together to promote late synthesis of PGE_2 (Fahmi, 2004). mPGES-2 is not well studied at present and its function is still unclear.

At transcriptional level, mPGES-1 expression is regulated by transcriptional factors such as early growth response protein-1 (Egr-1) and nuclear factor-kappa B (NF-κB) (Cheng et al., 2004;Ackerman et al., 2008). The regulation of mPGES-1 by Egr-1 was found through two Egr-1 binding motifs

(tandem GC boxes) that are located in the proximal region of the mPGES-1 promoter (Naraba et al., 2002;Subbaramaiah et al., 2004). On the other hand, mPGES-1 expression can be inhibited by a nuclear receptor called peroxisome proliferator-activated receptor gamma (PPARγ), which acts as a protector with anti-inflammatory and chondroprotective properties (Afif et al., 2007).

Recently, the characteristics, regulations and roles of mPGES-1 in inflammation have been studied in various cells, including vein endothelial cells, chondrocytes, synoviocytes. mPGES-1 has been identified to be a membrane bound microsomal enzyme, which is induced by glutathione and upregulated by proinflammatory stimuli, such as IL-1β, TNF-α and LPS at both mRNA and protein level (Stichtenoth et al., 2001;Uracz et al., 2002;Kojima et al., 2004).

5.3 Prostaglandin E_2 (PGE_2)

PGE_2 is an end product of AA through COX pathway. Its role as an inflammatory mediator is well known and its regulation mechanism is extensively studied. The group of Yucel-Lindberg investigated the effects of AA, PLA_2 and PGE_2 on the expression of mPGES-1 in fibroblasts. Interestingly, they found that $cPLA_2$ inhibitors downregulated mPGES-1 expression, which is upregulated by the addition of AA and PGE_2. These findings showed that the expression of mPGES-1 is regulated by PGE_2 through positive feedback regulation, amount of AA and functional $cPLA_2$ enzyme (Yucel-Lindberg et al., 2006). The effect of

PGE_2 on the enhancement of mPGES expression was also found in synovial fibroblasts from patients with RA (Kojima et al., 2003). Another study showed that PGE_2 has a regulatory function on the biosynthesis of leukotriene B_4 (LTB_4) and 5-LOX/FLAP expression. It decreases the production of LTB_4 by inhibiting FLAP via IL-10 dependent mechanism in dendritic cells, but without any effect on 5-LOX (Harizi et al., 2003). Recently, PGE_2 produced by synovial COX-2 was shown to be involved not only in inflammation, but also in the modulation of the degradation of cartilage proteoglycan mediated by IL-1β (Hardy et al., 2002).

5.4 5-lipoxygenase (5-LOX)

5-LOX is a pivotal enzyme that catalyzes the first two steps in the biosynthesis of LTs, including LTB_4 and cysteinyl-leukotrienes (cys-LTs). 5-LOX first catalyzes the conversion of AA to unstable epoxide intermediate LTA_4, which is subsequently transformed to either LTB_4 by LTA_4 hydrolase or cys-LTs by LTA_4 synthase **(Fig 7)**. These LTs are pro-inflammatory lipids mediators that are involved in asthma, inflammation, allergy and phagocyte chemotaxis (Wardlaw et al., 1989;Bigby, 1999).

5.5 Regulation of 5-LOX expression

5-LOX contains a large catalytic domain consisting of a non-heme iron atom and an ATP binding site, which are crucial for the catalytic potential of 5-LOX (Aparoy et al., 2008). Calcium and ATP are two important factors that

influence 5-LOX activation. In an environment without these two factors, the enzyme activity of 5-LOX is limited, but can be recovered by the addition of Ca^{2+} (Rouzer and Samuelsson, 1987). Interestingly, the increase in 5-LOX activity by ATP only happens in the presence of Ca^{2+} (Noguchi et al., 1996). Ca^{2+} plays an important role in the movement of 5-LOX to the nuclear membrane, allowing its binding with FLAP.

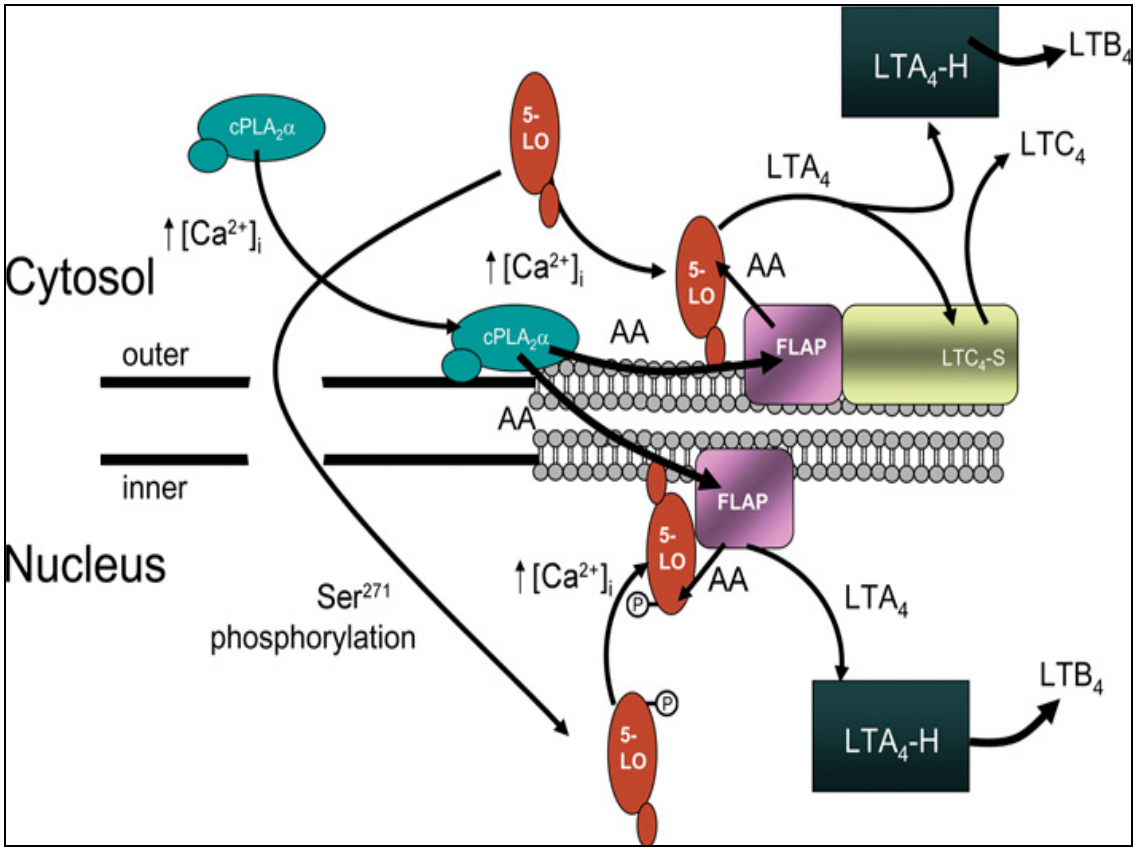
5-LOX is highly expressed in leukocytes, cancer cells and especially in differentiated cells. The expressions of 5-LOX mRNA and protein are induced by transforming growth factor-beta 1 (TGF-β1) and dihydroxyvitamin D_3, DMSO and granulocyte/macrophage colony-stimulating factor (GM-CSF) in human monocytic Mono Mac 6, leukemia HL-60 cells and granulocytes (Krump and Borgeat, 1994;Brungs et al., 1994). These findings suggested that TGF-β1, dihydroxyvitamin D_3 and GM-CSF act as differentiation inducers and promote 5-LOX synthesis in various cells (Krump and Borgeat, 1994;Harle et al., 1999). 5-LOX transcription activity increases when 5-LOX promoter construct is cotransfected with Egr-1 expression vector (Cheng et al., 2004). The transcription factor Egr-1 binds to GC sequences and interacts with transcriptional coactivators. This indicates that inducible 5-LOX gene transcription is mediated by Egr-1 and maybe by other transcriptional factors in GC rich promoter region (Silverman et al., 1998). 5-LOX expression can also be stimulated by dihydroxyvitamin D_3, which is synthesized in synovial fluid of patients with inflammatory arthritis. In OA cartilage, Tetlow and Woolley indicated that 5-LOX promoter contains a

vitamin D receptor element (VDRE), which induces 5-LOX expression by interaction with vitamin D receptors (VDR) (Tetlow and Woolley, 2001).

5.6 5-lipoxygenase-activating protein (FLAP)

FLAP is a nuclear membrane bound protein that collaborates with 5-LOX. When cells are stimulated, $cPLA_2$ and 5-LOX migrate to the nuclear membrane. FLAP helps the binding of 5-LOX with AA and the efficient utilization of AA released from phospholipids, leading to the biosynthesis of LTs **(Fig. 9)** (Murphy and Gijon, 2007). It has been shown that transformation of AA by 5-LOX is efficiently blocked in cells missing FLAP. Even FLAP is required for the LTs biosynthesis from endogenous AA, but metabolism of exogenous AA does not need FLAP.

This observation suggests that FLAP is not mandatory but can facilitate and enhance 5-LOX activity (Coffey et al., 1994). Furthermore, the expression of 5-LOX and FLAP as well as LTB_4 synthesis are enhanced in various inflammatory conditions, such as OA, systemic sclerosis and asthma (Chu et al., 2000;Kowal-Bielecka et al., 2001;Lascelles et al., 2009).



(Murphy RC, 2007)

Figure 9: Intracellular organization of the critical enzymes involved in leukotriene biosynthesis

6. OBJECTIVES AND HYPOTHESIS

Recently, we reported that the induction of COX-2 decreased dramatically after 8 hours of incubation with HNE, an end product of lipid peroxidation. HNE was thought to be involved in the pathophysiology of OA due to its ability to induce the expression of MMP-13 and COX-2, leading to destruction of cartilage and synovial inflammation. This study aimed to investigate the regulation of mPGES-1, FLAP and 5-LOX expression by HNE as well as the biosynthesis of PGE_2 and LTB_4 in human OA chondrocytes with different periods of incubation. We hypothesize that COX-2 and mPGES-1 down-regulation is attributed to HNE depletion and is responsible for the switch from COX-2 to FLAP/5-LOX pathway, resulting in the decrease of PGE_2 production and an increase of LTB_4. It has been shown that there is a switch of COX pathway to FLAP/5-LOX in the presence of low PGE_2. This study is also to investigate the involvement of transforming growth factor-beta 1 (TGF-β1) in this switch. In addition, increased LTB_4 level in synovial tissues was observed in OA patients who usually take COX-2 inhibitors. Therefore, our hypothesis is that HNE may be involved in the switch from COX-2 to FLAP/LOX pathway in human OA chondrocytes.

CHAPTER II:
MATERIALS AND METHODS

Specimen selection and chondrocyte culture

Human OA articular cartilage was obtained from OA patients aged 67 ± 9 years (mean ± SD) who underwent total knee arthroplasty. Informed consent had been obtained from patients with OA for the use of their tissues for research purposes. All patients were evaluated by rheumatologists who followed American College of Rheumatology criteria (Altman et al., 1986). The experimental protocol and use of human tissues were approved by the Research Ethics Board of Hôpital du Sacré-Cœur de Montréal. OA cartilage (femoral condyles and tibial plateaus) was obtained under aseptic conditions and carefully dissected from the underlying bone in each specimen (Benderdour et al., 2002). OA chondrocytes were extracted by sequential enzymatic digestion with 1 mg/ml of pronase (Sigma, Oakville, Ontario, Canada) for 1 h at 37ºC, then with 2 mg/ml of type IV collagenase (Sigma) for 6 h in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technology, Inc., Grand Island, NY, USA), 100 units/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen Life Technology, Inc.). The cells were seeded at high density in culture flasks at 37ºC in a humidified atmosphere of 5% CO_2/95% air until they were confluent and ready for the experiments. First-passage cells were employed to ensure their phenotype and seeded at 10^5 cells/cm^2 in culture tissue plates. The DMEM containing 2% FBS and antibiotics was replaced 24 h before the experiments were performed in this medium with the factors under study for different incubation time periods.

Chondrocytes were treated with single addition of 10 μM HNE for increasing times (0 to 72 h) or with repeated treatments by adding 10 μM HNE to the cultures at t=2, 4, 6, 8, 10, 12, 16, 24 h. For drug exposure, cells were pre-treated with 50 μM naproxen or 100 μg/ml anti-transforming growth factor-beta 1 (TGF-β1) antibody for 1 h, followed by a second incubation for 24 or 72 h in the presence or absence of 10 μM HNE.

Protein detection by Western blotting

Twenty micrograms of total proteins from chondrocyte lysates treated with HNE under the indicated conditions were loaded for discontinuous 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein transfer and immunodetection as well as semi-quantitative measurements were performed as described previously (Morquette et al., 2006). The primary antibodies were rabbit anti-COX-2, anti-mPGES-1 (Cayman Chemical-Cedarlane, Hornby, Ontario, Canada), anti-β-actin and anti-Egr-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After serial washes, primary antibodies were detected by goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Immunoreactive proteins were detected with SuperSignal blotting substrate (Pierce Biotechnology, Inc., Rockford, IL, USA) and exposed to clear-blue x-ray film (Pierce).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol reagent according to the manufacturer's instructions. RNA was quantitated with RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA), dissolved in diethylpyrocarbonate-treated H_2O, and stored at -80°C until used. One microgram of total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Fermentas, Burlington, Ontario, Canada), as detailed in the manufacturer's guidelines. One-fiftieth of the reverse transcriptase reaction product was analyzed by traditional PCR or real-time quantitative PCR. The following sense and antisense specific primers (purchased from Bio-Corp, Inc., Montreal, Quebec, Canada), were tested: human mPGES-1, 5'-GAA GAA GGC CTT TGC CAA C-3' (sense) and 5'-GGA AGA CCA GGA AGT GCA TC-3' (antisense); human 5-LOX, 5'-CTG TTC CTG GGC ATG TAC CC-3' (sense) and 5'-GAC ATC TAT CAG TGG TCG TG-3' (antisense); human FLAP, 5'-AAT GGG AGG AGC TTC CAG AG-3' (sense) and 5'-ACC AAC CCC ATA TTC AGC AG-3' (antisense); human GAPDH, 5'-CAG AAC ATC ATC CCT GCC TCT-3' (sense) and 5'GCT TGA CAA AGT GGT CGT TGA G-3' (antisense).

Quantitative PCR analysis was performed in a total volume of 50 µl containing template DNA, 200 nM sense and antisense primers, 25 µl of SYBR Green Master Mix (Qiagen, Mississauga, Ontario, Canada), and 0.5 units of uracil-

N-glycosylase (UNG; Epicentre Technologies, Madison, WI, USA). After incubation at 50°C for 2 min (UNG reaction) and at 95°C for 10 min (UNG inactivation and activation of AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 s at 95°C for denaturation and 1 min for annealing and extension at 60°C). Incorporation of SYBR Green dye into the PCR products was monitored in real time with a Mx3000 real-time PCR systems (Stratagene, La Jolla, CA, USA), to determine the threshold cycle (C_t) at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with 1 peak, indicating amplification specificity. A C_t value was obtained from each amplification curve with the software provided by the manufacturer (Stratagene).

Relative mRNA expression in chondrocytes was quantified according to the $\Delta\Delta C_t$ method, as detailed in the manufacturer's guidelines (Stratagene). A ΔC_t value was first calculated by subtracting the C_t value for the housekeeping gene GAPDH from the C_t value for each sample. A $\Delta\Delta C_t$ value was then calculated by subtracting the ΔC_t value for the controls (unstimulated cells) from the ΔC_t value for each treatment. Fold changes compared with the controls were then determined by $2^{-\Delta\Delta C}{}_t$. Each PCR generated only the expected specific amplicon, as shown by melting temperature profiles of the final product and gel electrophoresis of the test PCRs. Each PCR was performed in triplicate on 2 separate occasions for each independent experiment.

PGE_2, LTB_4 and transforming growth factor-beta 1 (TGF-β1 enzyme immunoassay

After incubation, the culture medium from cultured OA chondrocytes was collected and PGE_2 and LTB_4 levels were measured with specific commercial kits for PGE_2 (Cayman Chemical), LTB_4 and TGF-β1 (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Detection sensitivity was 9, 13.7, 4.6 pg/ml, respectively. All assays were in triplicate.

Plasmids and transient transfection

The human mPGES-1 promoter construct (-538/-28) was a gift from Dr. Terry J. Smith (University of California, Los Angeles, CA, USA) (Han et al., 2002). The pEgr-1Mutx3-TK-Luc reporter construct was generously provided by Dr. Yuqing E. Chen (Morehouse School of Medicine, Atlanta, GA, USA) (Fu et al., 2002). The 5-LOX promoter construct was kindly donated by Dr. Dieter Steinhilber (University of Frankfurt, Frankfurt, Germany). Subconfluent human OA chondrocytes were transiently transfected in 12-well cluster plates with lipofectamine 2000^{TM} reagents (Invitrogen Life Technology, Inc.), according to the manufacturer's protocol. Briefly, transfections were conducted for 6 h with DNA lipofectamine complexes containing 10 µl of lipofectamine reagent, 2 µg DNA plasmid and 0.5 µg of pCMV-β-gal (as a transfection efficiency control). After washing, the cells were stimulated with 10 µM HNE for different incubation periods, depending on the experiments. Luciferase activity was determined in

cellular extracts with commercially-available kits (Luciferase assay system, Promega Corporation, Madison, WI, USA). The data were normalized to β-gal level, which was quantified by a specific ELISA (Roche Applied Science, Laval, Quebec, Canada).

Statistical analysis

For the time course experiments, all quantitative results were expressed as means ± SEM. The data were compared by ANOVA. *p* values less than 0.05 were considered to be statistically significant and contrast tests were against the control (time = 0 h). Two-way ANOVA was used to analyze the results from the experiments with treatments of two factors, such as HNE and naproxen or HNE and TGF-β1. Their interaction and main effects are reported in the figures.

CHAPTER III: RESULTS

Single or repeated treatments with HNE regulate differently PGE_2 release and COX-2 protein expression

As described in our previous report, COX-2 expression decreased gradually after 8 h of chondrocyte incubation with 10 μM HNE. To examine whether this reduction is attributable to HNE depletion, cells were stimulated with either one or repeated 10 μM HNE treatments for different incubation periods (0 to 24 h, n = 3 independent experiments). Chondrocyte stimulation with 10 μM HNE (1 treatment) showed that COX-2 protein expression and PGE_2 release increased within 4 h and plateaued at 8 h of incubation, before declining at 16 and 24 h (Fig. 1A & 1C). PGE_2 and COX-2 reached maximum levels of 157.5 ± 33 ng/10^5 cells and 340% of control values, respectively. However, in experiments where the cells were stimulated repeatedly, COX-2 expression and PGE_2 release were enhanced after 4 h and 8 h of incubation respectively, with maximum values obtained at 24 h (Fig. 1B & 1D). Maximum PGE_2 and COX-2 values were 210 ± 29 ng/10^5 cells and 400% of control values, respectively. HNE was cytotoxic at >20 μM and significantly decreased cell viability (data not included).

Induction of mPGES-1 protein and mRNA expression by single or repeated treatment with HNE

To gain insights into the role of HNE in the modulation of mPGES-1, the inducible form of PGES involved in the terminal step of PGE_2 synthesis, we

Figure 10

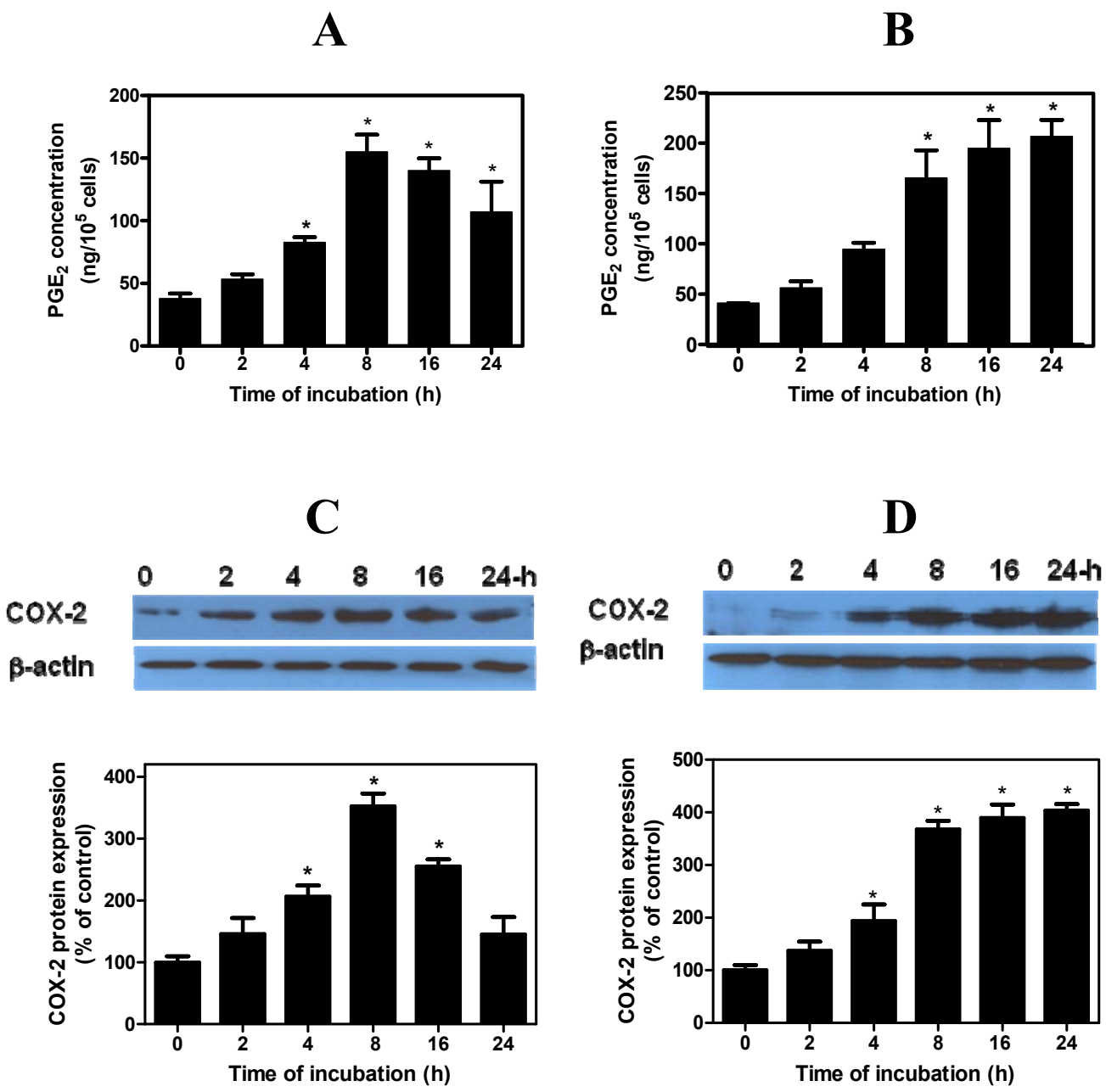

A
B
C
D
PGE2 concentration (ng/10^5 cells)
Time of incubation (h)
0 2 4 8 16 24
0 2 4 8 16 24-h
COX-2
β-actin
COX-2 protein expression (% of control)
*

Figure 10. HNE-induced PGE_2 production and COX-2 expression in human OA chondrocytes

Cells were stimulated once (A & C) or repeatedly (B & D) with 10 μM HNE every 2 h for different incubation periods, as indicated above. PGE_2 concentration (A & B) was measured by enzyme immunoassay (EIA) commercial kit. COX-2 protein expression (C & D) was analyzed by Western blotting. The data are means ± SEM of n = 3 and contrast test is against the control (time = 0 h). The significance was calculated by ANOVA: * $p < 0.05$.

evaluated its effect on the expression of this enzyme in human OA chondrocytes treated once or several times for different incubation periods (n = 3 independent experiments). As depicted in Figures 2A and 2C, one treatment with 10 μM HNE significantly increased mPGES-1 protein and mRNA levels, which plateaued at 16 h but then declined at 24 h. mPGES-1 protein and mRNA reached maximum levels of 230 and 214% of control values, respectively. However, repeated treatments with 10 μM HNE enhanced mPGES-1 protein and mRNA levels, with maximum value attained at 24 h (Fig. 2B and 2C). The maximum levels of mPGES-1 protein and mRNA were 430 and 300% of the controls, respectively.

HNE-induced mPGES-1 and 3xEgr-1 binding site promoter activities

In our previous report, we have reported that HNE induced COX-2 transcription through ATF-2/CREB-1 transactivation (Vaillancourt et al., 2007). In the present study, we further examined whether HNE induced mPGES-1 at transcriptional level through Egr-1 transactivation. Firstly, to determine whether changes in mPGES-1 mRNA levels can be ascribed to alteration in promoter activity, chondrocytes were transiently transfected with human mPGES-1 promoter-luciferase reporter genes (n = 3 independent experiments). Treatment of transfected cells with 10 μM HNE for increasing periods of incubation led to a time-dependent increment of mPGES-1 promoter activity (Fig. 3A). Maximum promoter activity was 331% at 16 h compared to the controls. These data are

Figure 11

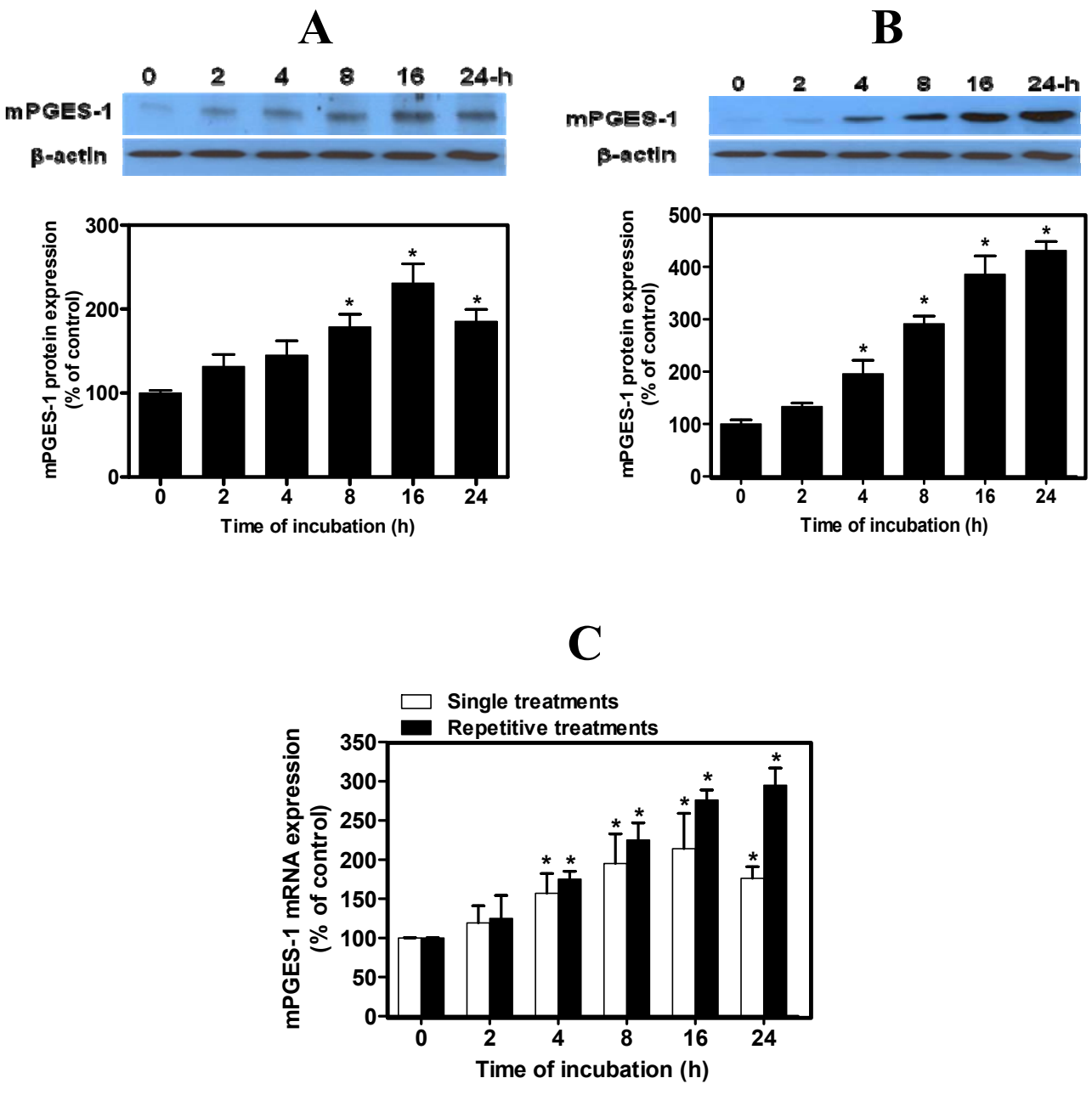

A
0 2 4 8 16 24-h
mPGES-1
β-actin
mPGES-1 protein expression (% of control)
Time of incubation (h)
B
0 2 4 8 16 24-h
mPGES-1
β-actin
mPGES-1 protein expression (% of control)
Time of incubation (h)
C
Single treatments
Repetitive treatments
mPGES-1 mRNA expression (% of control)
Time of incubation (h)

Figure 11. HNE-induced mPGES-1 protein and mRNA expression in human OA chondrocytes

Cells were stimulated with 10 μM HNE once (A & C) or repeatedly (B & C) with 10 μM HNE every 2 h for different incubation periods, as indicated above. mPGES-1 protein (A & B) and mRNA (C) were measured respectively by Western blotting and real-time RT-PCR. The data are means ± SEM of n = 3 and contrast test is against the control (time = 0 h). The significance was calculated by ANOVA: * $p < 0.05$.

Figure 12

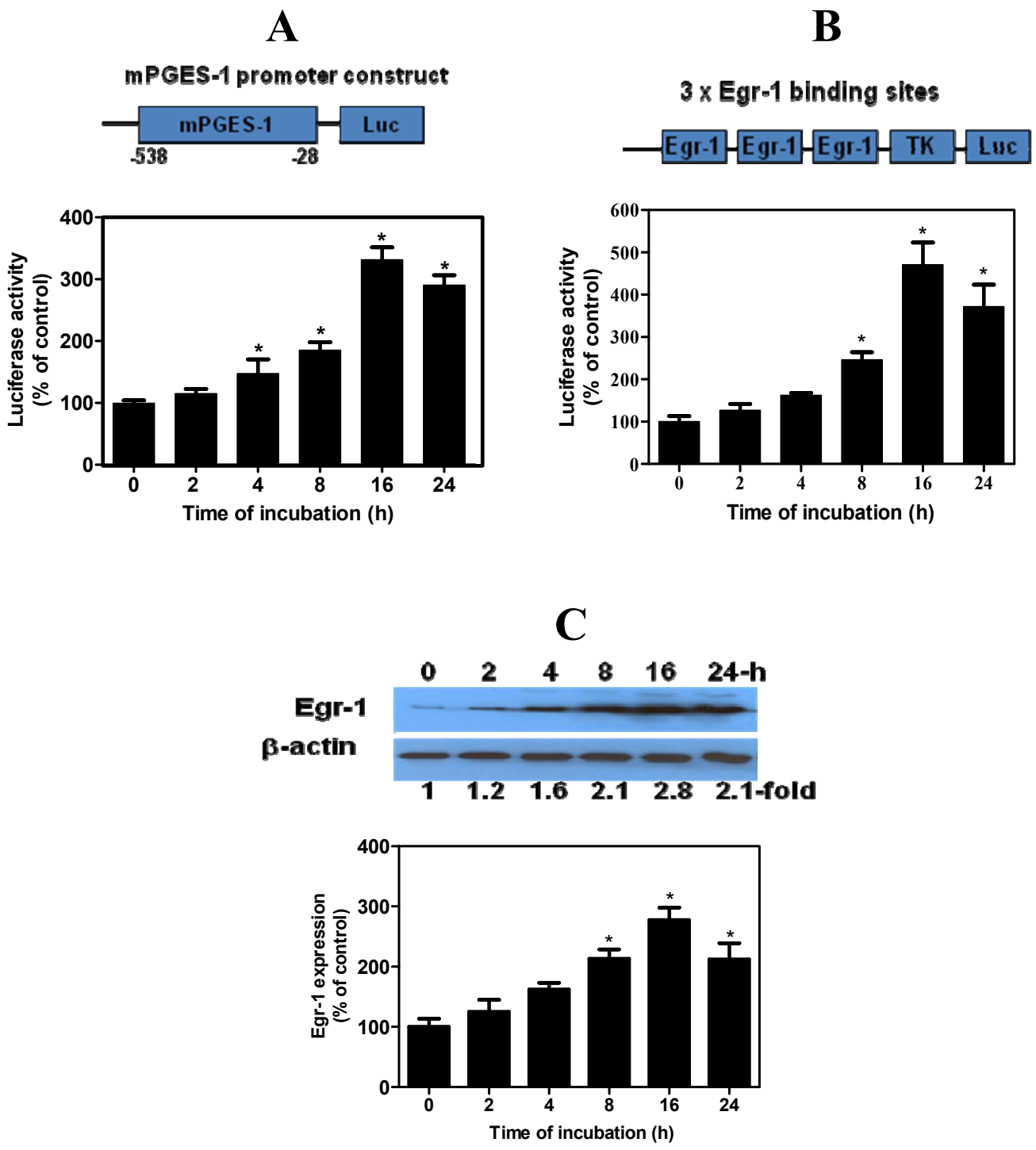

A
mPGES-1 promoter construct
mPGES-1
Luc
-538
-28
Luciferase activity (% of control)
Time of incubation (h)
B
3 x Egr-1 binding sites
Egr-1
Egr-1
Egr-1
TK
Luc
Luciferase activity (% of control)
Time of incubation (h)
C
0 2 4 8 16 24-h
Egr-1
β-actin
1 1.2 1.6 2.1 2.8 2.1-fold
Egr-1 expression (% of control)
Time of incubation (h)

Figure 12. HNE-activated mPGES-1 promoter

Chondrocytes were transfected with mPGES-1-Luc (A) or p3xEgr-1-Luc (B) containing 3 Egr-1 binding sites. The next day, they were incubated with 10 μM HNE for increasing incubation time periods. Luciferase activity was measured in cell extracts and normalized to β-galactosidase activity. (C) OA chondrocytes were treated with 10 μM HNE at increasing time of incubation and then Egr-1 protein expression was evaluated in cellular extract by Western blot. The data are means ± SEM of n = 3 and contrast test is against the control (time = 0 h). The significance was calculated by ANOVA: * $p < 0.05$.

consistent with the regulation of mPGES-1 expression by HNE at the transcription level. The transcription factor Egr-1 has been shown to play a crucial role in mPGES-1 transcription. Therefore, we analyzed the effect of HNE on the transcriptional activation of a synthetic luciferase reporter construct containing 3 tandem repeats of the putative Egr-1 binding sequence pEgr-1×3-TK-Luc. As seen in Figure 3B, HNE increased luciferase activity of the above construct, and this activation was time-dependent. Maximum promoter activity reached 450% at 16 h compared to the controls (Fig. 3B). Western blot analysis revealed that single treatment with 10 μM HNE induced Egr-1 protein expression by 280% compared to untreated cells after 16 h of incubation (Fig. 3C). Collectively, these data suggest that Egr-1 may be one of the transcription factors involved in HNE-induced mPGES-1 promoter activity.

HNE-induced LTB_4 release through 5-LOX and FLAP up-regulation

LTB_4 is an end product of an inflammatory signaling pathway. It has been found to regulate the synthesis of other catabolic factors involved in the pathophysiology of OA (Martel-Pelletier et al., 2004). Human OA chondrocytes (n = 5 independent experiments) were stimulated with 10 μM HNE for different incubation periods. HNE-induced changes in LTB_4 level was measured with EIA commercial kits. LTB_4 release (Fig. 4A) was induced only after 48 h incubation period, with a further increase after 72 h of treatment (66.6 ± 3.7 pg/10^5 cells).

Figure 13

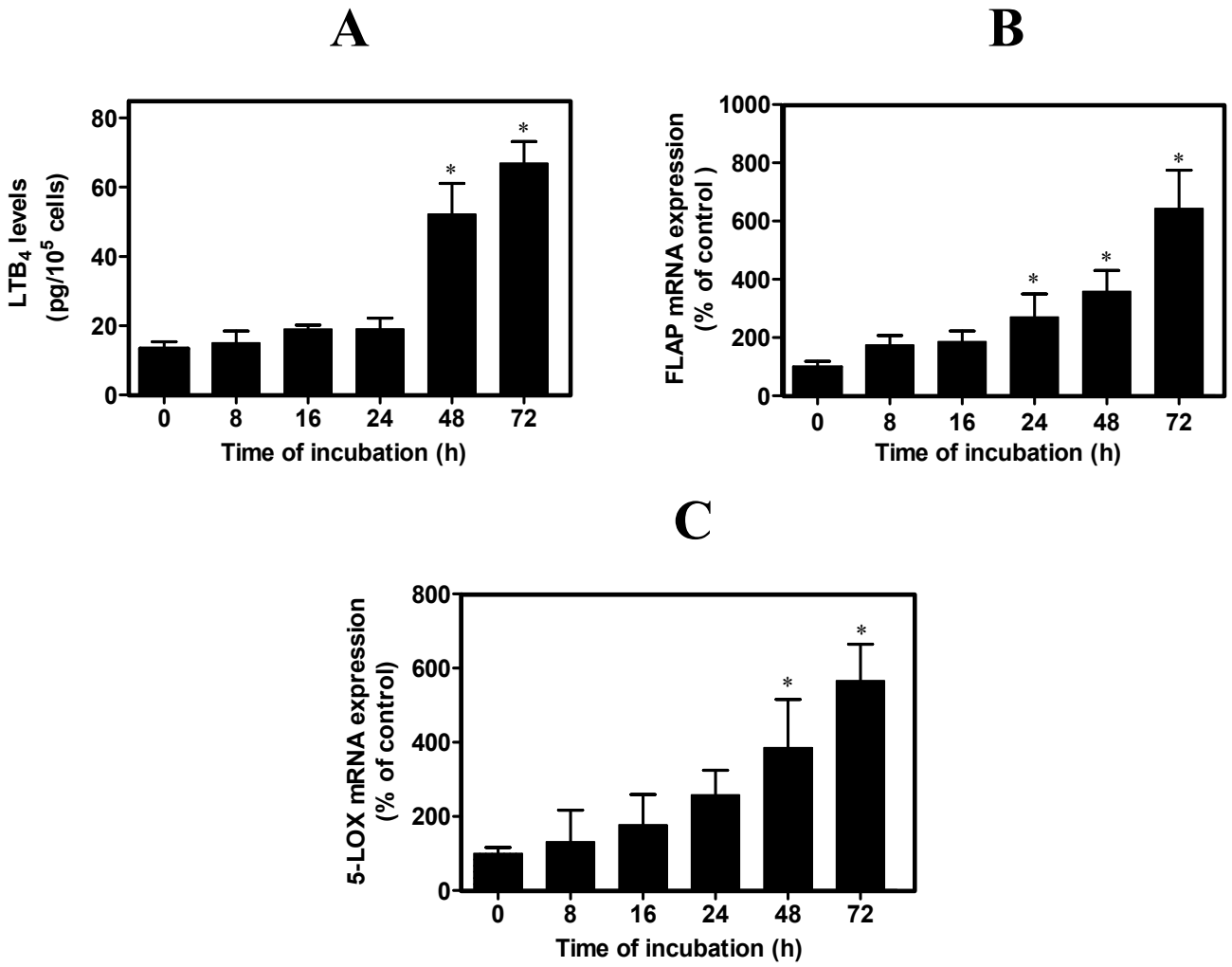


Figure 13. HNE-induced LTB_4 biosynthesis and 5-LOX and FLAP mRNA expression in human OA chondrocytes

Cells were treated with 10 μM of HNE for different incubation time periods, as indicated above. LTB_4 production (A) was evaluated by EIA commercial kit. FLAP (B) and 5-LOX (C) mRNA expression was measured by real-time RT-PCR. The data are means ± SEM of n = 5 and contrast test is against the control (time = 0 h). The significance was calculated by ANOVA: * $p < 0.05$.

5-LOX and FLAP are essential proteins responsible for LTB_4 synthesis. To determine whether augmented LTB_4 production induced by HNE is related to 5-LOX and FLAP synthesis, we further examined the effect of HNE on their mRNA expression. As shown in Figures 4B and 4C, our data disclosed that both FLAP and 5-LOX mRNA expression rose significantly but differentially after incubation in human OA chondrocytes. FLAP mRNA expression occurred earlier, after 24 h of incubation, compared to 5-LOX (48 h). Maximum FLAP and 5-LOX levels were 644% and 566% of control values, respectively.

HNE-induced 5-LOX promoter activity

To more accurately identify whether HNE-induced 5-LOX at the transcription level, the 5-LOX promoter construct with luciferase gene reporter was transiently transfected into human OA chondrocytes, followed by stimulation with 10 μM HNE (n = 3 independent experiments). Compared to unstimulated cells, HNE induced 5-LOX gene promoter activity in transfected chondrocytes. 5-LOX promoter activity increased significantly by 310% and 580% after 48 and 72 h of incubation, respectively (Fig. 5). These data indicated that HNE regulates 5-LOX mRNA expression at transcriptional level.

Figure 14

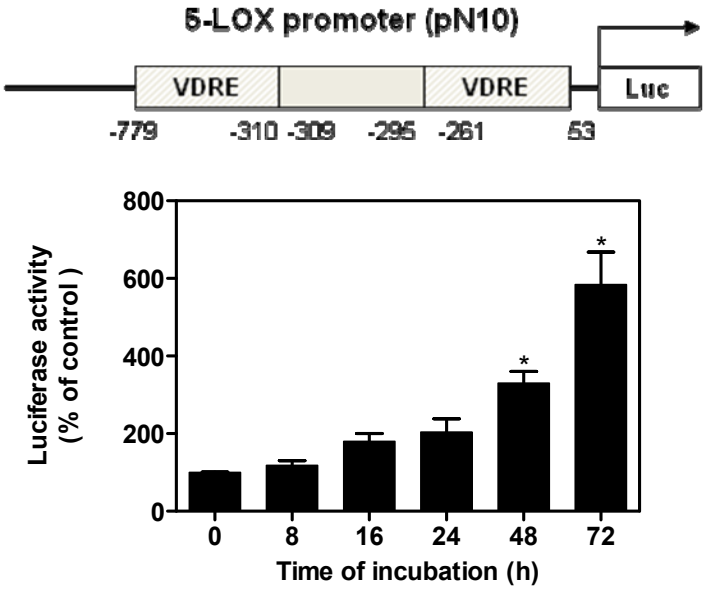


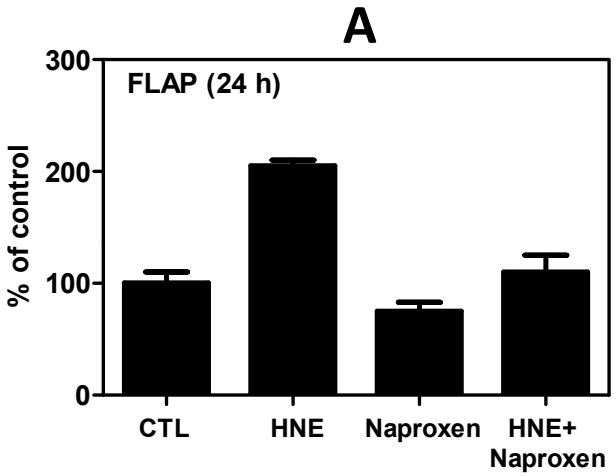
Figure 14. HNE-activated 5-LOX promoter in human OA chondrocytes

Cells were transfected with human 5-LOX promoter. The next day, they were incubated with 10 μM HNE for increasing incubation time periods. Luciferase activity was measured in cell extracts and normalized to β-galactosidase activity. The data are means ± SEM of n = 3 and contrast test is against the control (time = 0 h). The significance was calculated by ANOVA: * $p < 0.05$.

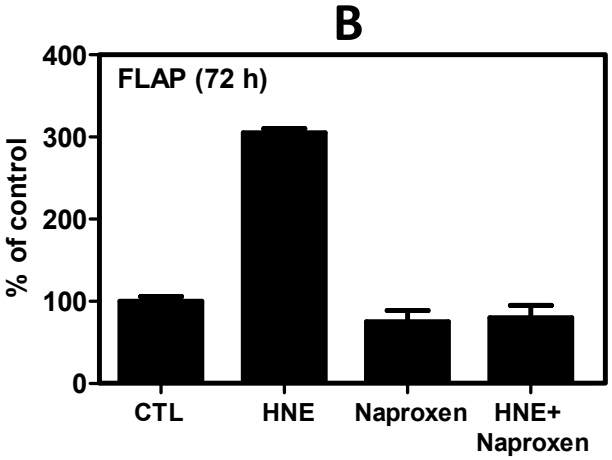
Effect of COX inhibition on FLAP and 5-LOX expression

Further experiments were aimed at elucidating the role of PGE_2 in HNE-induced 5-LOX and FLAP expression in OA chondrocytes. Firstly, cells were treated with 50 μM naproxen (a non-selective COX inhibitor) for 1 h followed by another treatment of 10 μM HNE for 24 or 72 h (n = 3 independent experiments). Our real-time RT-PCR results demonstrated that HNE induced FLAP expression after 24 h of treatment (210% of control) and the level was even higher after 72 h (315% of control) (Fig. 6A and 6B). In contrast, FLAP expression was diminished by naproxen after 24 and 72 h (75% of control). HNE-induced FLAP expression was also abolished by naproxen, which prevents PGE_2 production. HNE did not induce 5-LOX expression after 24 h of incubation. It stimulated 5-LOX expression only after 72 h by 600% above control values (Fig. 6D). However, incubation of cells with naproxen caused a consistent increase of 5-LOX mRNA level by 330% and 570% of control values after 24 and 72 h of incubation, respectively (Fig. 6C & 6D). We observed that PGE_2 has different effects on the expression of FLAP and 5-LOX. It has been indicated that the inhibition of PGE_2 up-regulates 5-LOX while down-regulating FLAP gene expression (Martel-Pelletier et al., 2004). We showed that the production of PGE_2 during 24 h of HNE incubation prevents 5-LOX expression. However, between 24 and 72 h, the level of PGE_2 (35 ± 15 (control) vs. 55 ± 21 (72 h), not significant) was low due to the decrease in COX-2 expression, which allows 5-LOX to be expressed (Fig. 4C). All these data suggest that PGE_2 has opposite effects on 5-LOX and FLAP regulation by HNE.

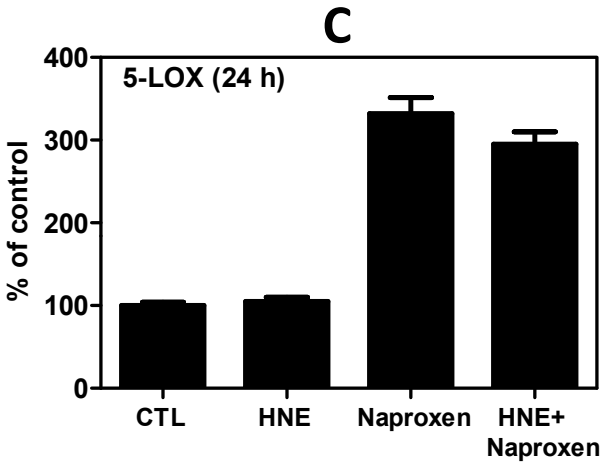
Figure 15



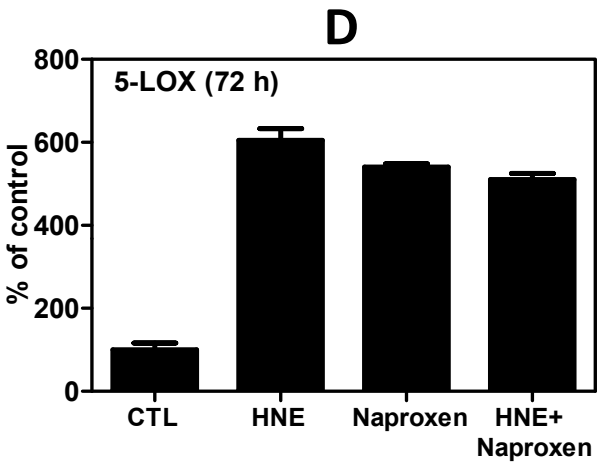
Two-way ANOVA
HNE: $F(1,8) = 47.1$, $p < 0.05$
Naproxen: $F(1,8) = 34.9$, $p < 0.05$
HNE + Naproxen: $F(1,8) = 11.7$, $p < 0.05$



Two-way ANOVA
HNE: $F(1,8) = 92.1$, $p < 0.05$
Naproxen: $F(1,8) = 130.6$, $p < 0.05$
HNE + Naproxen: $F(1,8) = 83.6$, $p < 0.05$



Two-way ANOVA
HNE: $F(1,8)$ = NS
Naproxen: $F(1,8) = 272.3$, $p < 0.05$
HNE + Naproxen: $F(1,8)$ = NS



Two-way ANOVA
HNE: $F(1,8) = 167.1$, $p < 0.05$
Naproxen: $F(1,8) = 88.2$, $p < 0.05$
HNE + Naproxen: $F(1,8) = 212.0$, $p < 0.05$

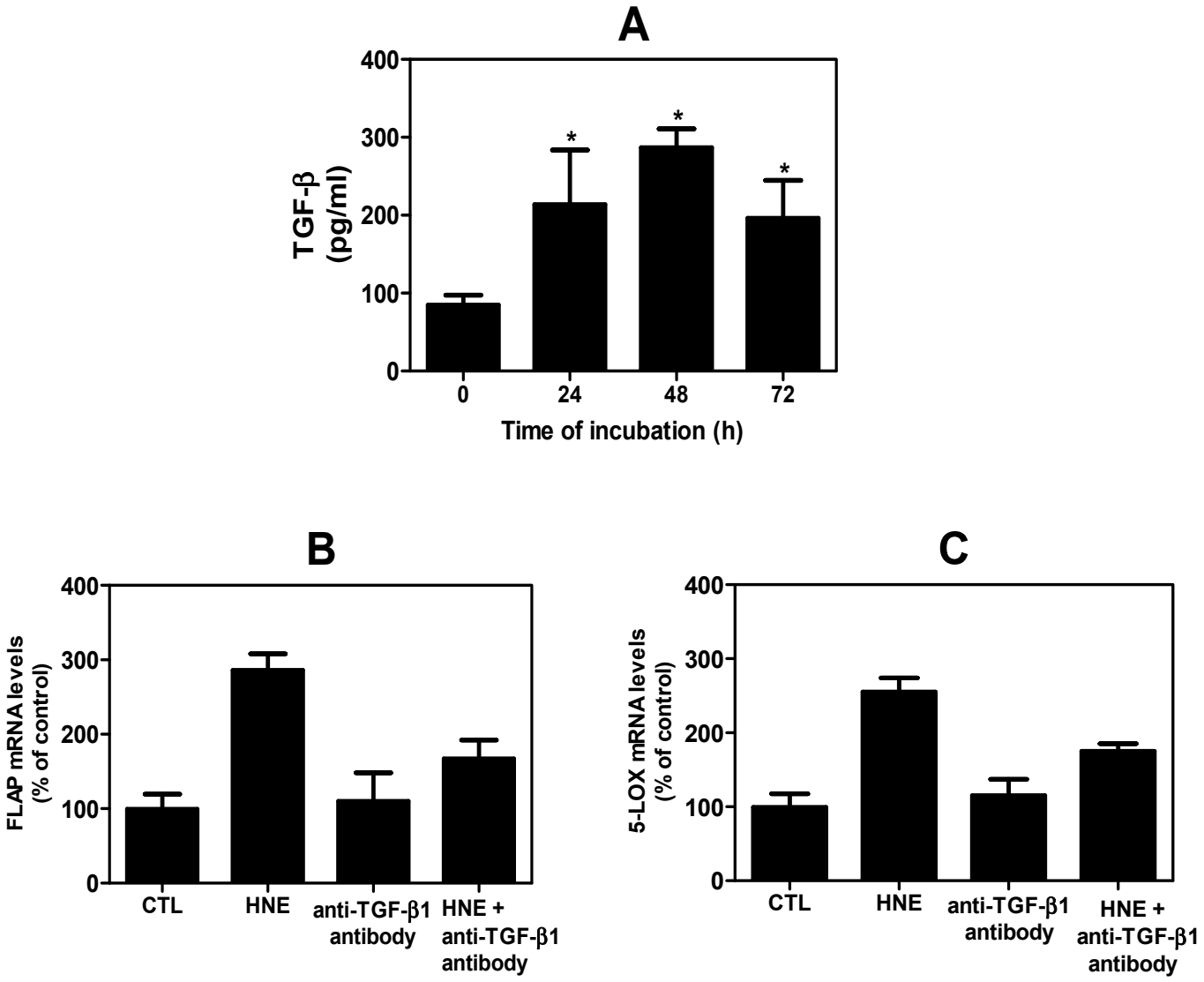
Figure 15. HNE-induced FLAP/5-LOX expression is PGE_2-dependent in human OA chondrocytes

Cells were treated with 50 μM naproxen, 10 μM HNE or both for 24 or 72 h. FLAP (A & B) and 5-LOX (C & D) mRNA expression was evaluated by real-time RT-PCR. The data are means ± SEM of n = 3 and contrast test is against the control (time = 0 h). The significance was calculated by ANOVA.

HNE induction of 5-LOX/FLAP mRNA is TGF-β1-dependent

In other experiments, we determined whether TGF-β1 production could be involved in HNE-induced 5-LOX/FLAP expression. Chondrocytes were treated or not with 10 μM HNE for 72 h in the presence or absence of 100 μg/ml of TGF-β1 antibody. As illustrated in Figure 7, 10 μM HNE elevated TGF-β1 levels in chondrocyte culture media (n = 3 independent experiments) to 210, 288 and 201 pg/ml at 24, 48, 72 h of incubation respectively. Interestingly, the addition of anti-TGF-β1 antibody (TGF-β1 neutralization) to cultured cells attenuated HNE-induced 5-LOX and FLAP by 50-65%, from 255% to 175% for 5-LOX mRNA and from 286% to 167% for FLAP mRNA expression. It has been showed that TGF-β1 induced a marked enhancement in FLAP and 5-LOX mRNA levels (Martel-Pelletier et al., 2004). Our data suggest that 5-LOX and FLAP regulation by HNE is partially mediated via TGF-β1 production.

Figure 16



Two-way ANOVA
HNE: $F(1,8) = 11.9$, $p < 0.05$
Anti-TGF-β1 Ab: $F(1,8) = 60.3$, $p < 0.05$
HNE + Anti-TGF-β1 Ab: $F(1,8) = 17.1$, $p < 0.05$

Two-way ANOVA
HNE: $F(1,8) = 9.8$, $p < 0.05$
Anti-TGF-β1 Ab: $F(1,8) = 109.8$, $p < 0.05$
HNE + Anti-TGF-β1 Ab: $F(1,8) = 21.6$, $p < 0.05$

Figure 16. HNE-induced FLAP/5-LOX expression is TGF-β1-dependent in human OA chondrocytes

TGF-β1 level (A) was measured with ELISA commercial kits and expressed as pg/ml. (B & C) Cells were treated with 10 μM HNE for 72 h in the presence or absence of anti-TGF-β1 antibody, 5-LOX and FLAP mRNA levels were then assessed by real-time RT-PCR. The data are means ± SEM of n = 3 and contrast test is against the control (time = 0 h). The significance was calculated by ANOVA: * $p < 0.05$.

CHAPTER IV: DISCUSSION

HNE: A pathophysiological modulator

Numerous studies have shown that oxidative stress was involved in the pathophysiology of OA, caused by the excessive production of ROS and an insufficient availability of antioxidants to detoxify ROS (Chen et al., 2008;Davies et al., 2008;Roman-Blas et al., 2009). ROS induces LPO of membrane polyunsaturated fatty acids, leading to the production of aldehydes from AA. Similar to free radicals, aldehydes are eletrophiles that bind to the nucleophilic groups of proteins, (amino)phospholipids and nucleic acids, but their relatively longer half-life made them candidates for the propagation of damage to neighbouring cells. Among the aldehydes, HNE is considered to be the most reactive species because of its α,β-double bond, which make HNE more susceptible to nucleophilic attack (Uchida, 2003). Whether aldehydes are "second toxic messengers", as first hypothesized by Esterbauer (Esterbauer et al., 1991), or merely represent changed markers of extensive tissue damage (Halliwell and Chirico, 1993), has long been a subject of controversies. This situation recently changed, when HNE was found to be a normal constituent of mammalian tissue membranes (Poli et al., 2008). One specific property of HNE is its ability to bind to proteins. The α,β-double bond of HNE reacts readily with specific amino acid and coenzyme residues of proteins via Michael-type addition: the sulfhydryl group of cysteine (CYS) or lipoic acid, the ε-amino group of lysine (LYS), or the imidazol function of histidine (HIS).

HNE occupies a prominent place in the hierarchy of LPO end-products and growing evidence supports it as a (patho)physiological modulator of signal transduction, transcriptional regulation, and post-translational modification of proteins (Uchida, 2003;Poli et al., 2008). It is found to be involved in many diseases where oxidative stress is increased, including atherosclerosis, neurodegenerative disorders and cancers (Cerbone et al., 2007;Mattson, 2009). The relevance of HNE to joint biology and pathology is now becoming clearer. In our previous study, we have reported for the first time that the level of HNE was significantly increased in synovial fluids of patients with OA compared to normal subjects and in OA chondrocytes and osteoblasts treated with ROS donors (Morquette et al., 2006;Shi et al., 2006). These data are consistent with the results reported by Grigolo and colleagues, (Grigolo et al., 2003) who found higher levels of both malondialdehyde (MDA) and HNE in human OA synoviocytes compared to normal cells. Furthermore, we demonstrated that HNE induced transcriptional and post-translational modification of Col II and MMP-13, suggesting that HNE could play a role in cartilage degradation in OA (Morquette et al., 2006). In another study, we have identified two distinct mechanisms of action of HNE in OA chondrocytes, which include upregulation of COX-2 via ATF/CREB activation and inhibition of iNOS via NF-κB inactivation (Vaillancourt et al., 2007). In this previous study, we showed that COX-2 expression at protein and mRNA levels was detectable within 2 h of incubation with 10 μM of HNE and plateaued at 8 h of incubation before declining afterwards. Moreover, we revealed

that HNE-induced COX-2 production expression required the activation of p38 MAPK signaling pathway. Furthermore, overexpression of WT p38 MAPK in OA chondrocytes enhanced HNE-induced COX-2 protein expression and promoter activity. Interestingly, this induction is specific for HNE since other aldehydes, such as acrolein and 2-nonenal, possessing an analogous functionality to HNE were all inactive in terms of COX-2 expression in these cells (Kumagai et al., 2000).

Regulation of COX-2 and mPGES-1 by HNE

In the present study, we have extended our investigation to evaluate the potential role of HNE in inflammatory response in OA. With the ultimate goal of clarifying this role, we documented firstly the ability of HNE to modulate COX-2 and mPGES-1 as well as the production of PGE_2, using two types of stimulation: single and repeated treatments. We have observed that 10 μM HNE (with one treatment) induced PGE_2 release as well as COX-2 and mPGES-1 expression at protein and mRNA levels and plateaued at 8 h of incubation before declining afterwards at 16 and 24 h respectively in OA chondrocytes. These data are in concordance with our previously reported COX-2 data and are supported by other studies indicating that HNE-induced COX-2 expression and PGE_2 release declined rapidly in various cell lines, such as rat liver RL34 epithelial cells and RAW264.7 macrophages (Kumagai et al., 2000;Kumagai et al., 2002;Kumagai et al., 2004;Vaillancourt et al., 2007). Interestingly, our results indicated that the

reductions of COX-2 and mPGES-1 expression at 16 h and 24 h were completely abolished by repetitive stimulation of cells with 10 μM HNE (8 treatments at regular interval of 2 h), supporting the hypothesis that the decrease of COX-2 and mPGES-1 expression is linked to HNE metabolism in chondrocytes. Mammalian cells have developed multiple enzymatic pathways for the detoxification of HNE. The best characterized of these enzymes include the aldehyde dehydrogenase and alcohol dehydrogenase (Esterbauer et al., 1991;Ullrich et al., 1997). The estimated half-life of HNE in cells was approximately 1 h (Pizzimenti et al., 2002).

Since the regulation of mPGES-1 by HNE had not yet been reported, we extended our investigation to determine whether changes in mRNA levels can be ascribed to changes in promoter activity. To do so, human OA chondrocytes were transiently transfected with the human mPGES-1 promoter-luciferase reporter gene. Treatment of transfected cells with 10 μM HNE at increasing periods of incubation led to a time-dependent increase of the mPGES-1 promoter activity. These data are consistent with the regulation of mPGES-1 expression by HNE at the transcriptional level. The mPGES-1 promoter construct contains several proximal regulation sites, such as Egr-1 and NF-κB (Cheng et al., 2004; Ackerman et al., 2008). Emerging evidence suggests that the transcriptional induction of mPGES-1 by cytokines and growth factors is controlled primarily by Egr-1 through two Egr-1 binding motifs identified in the proximal promoter region of the mPGES-1 region (Cheng et al., 2004). Thus, we hypothesized that induction of

Egr-1 activity by HNE could be the mechanism by which HNE exerts its inducer effect on mPGES-1 transcription. To gain insight into the mechanism of HNE-mediated mPGES-1 expression through Egr-1 activation, OA chondrocytes were transiently transfected with the synthetic luciferase reporter construct containing three tandem repeats of Egr-1 motif. Data showed that HNE induced the transcriptional activity of the above construct, suggesting the implication of this transcription factor in HNE-induced transcriptional activity of mPGES-1 promoter. In our Western blot analysis, we observed that 10 μM HNE increased Egr-1 protein expression in a time-dependent manner. Recently, ROS were reported to enhance Egr-1 expression, which in turn contributes to the elevated level of mPGES-1 in inflamed cells (Hasan and Schafer, 2008). Cheng et al. 2004 examined the role of PPARγ in the regulation of mPGES-1 in OA cartilage; the authors showed that PPARγ activation suppresses mPGES-1 expression via negative interference with Egr-1. Altogether, this is the first evidence supporting that mPGES-1 up-regulation by HNE required Egr-1 transcriptional activity and expression in OA chondrocytes.

Regulation of FLAP and 5-LOX by HNE

FLAP and 5-LOX are two primary enzymes involved in LTB_4 biosynthesis. Moreover, recent investigations of how FLAP activates 5-LOX propose that 5-LOX does not actually bind FLAP, but rather that FLAP binds arachidonic acid and presents it to 5-LOX in such a manner that 5-LOX becomes fully

enzymatically active (Peters-Golden, 1998). The expressions of 5-LOX and FLAP have been variably documented in different cells and tissues. Usually, significant levels are found in bone marrow-derived cells, such as neutrophils, dendritic cells and some lymphocytes but 5-LOX and FLAP levels are not detectable in undifferentiated cells (Peters-Golden and Brock, 2003). 5-LOX and FLAP are highly enhanced by TGF-β1 and dihydroxyvitamin D_3, followed by increased production of LTB_4 (Brungs et al., 1995;Martel-Pelletier et al., 2004). We deciphered the role of HNE in 5-LOX and FLAP expression in order to determine the mechanism underlying the modulation of LTB_4 biosynthesis in human OA chondrocytes.

Our data suggested a potential 5-LOX and FLAP up-regulation by HNE, occurring at least in part, at the transcriptional level, as determined by real-time quantitative RT-PCR and transient transfection experiments. HNE-induced both FLAP and 5-LOX mRNA expression in a time-dependent but different fashion. The expression of FLAP is activated earlier than that of 5-LOX, which occurs only after 48 hours of HNE stimulation. In the same pattern as 5-LOX expression, HNE enhanced LTB_4 release after 48 and 72 h of incubation. However, our attempts to detect 5-LOX and FLAP protein by Western blot in total cellular protein extracts were unsuccessful probably because of their low level as indicated by Martel-Pelletier et al., 2004. Thereafter, to determine whether the regulation of 5-LOX mRNA by HNE occurred at transcriptional level, we carried out transient transfection studies. Human OA chondrocytes were transfected with a human 5-

LOX promoter (–779 to +53) region/luciferase reporter gene construct and stimulated with 10 μM HNE at different times of incubation. Data showed that HNE increased the luciferase activity of the 5-LOX promoter only after 48 hours of incubation. The 5-LOX promoter sequence contains consensus binding sites for several transcriptional factors, including Sp1, Egr-1, VDR, NF-κB and AP family members. The FLAP promoter sequence contains a TATA box and glucocorticoid receptor binding sites (Peters-Golden and Brock, 2003). The investigation of the activation of the transcriptional factors associated with these promoters can help us to gain insight into how HNE regulates 5-LOX and FLAP expression at transcriptional level.

These results were consistent with a previous report of Martel-Pelletier et al., 2004 showing that 5-LOX expression is activated in late fashion, leading to the late increase in LTB_4 production (Martel-Pelletier et al., 2004). The authors showed that FLAP mRNA level was significantly enhanced after short period (20 h) of treatment with TGF-β1 and 1,25-dihydroxyvitamin D_3, whereas 5-LOX mRNA level increased only after 72 h (Martel-Pelletier et al., 2004). These authors suggested that the reason for the late increase in 5-LOX mRNA may be that TGF-β1 induces only an accumulation of 5-LOX mRNA and not a true up-regulation of the gene expression per se. The increase in LTB_4 production upon HNE stimulation occurred late (48 h) after the beginning of the stimulation. This coincided with the time at which the 5-LOX mRNA level was increased and the

FLAP gene expression remained high. These data confirm that LTB_4 production depends on the regulation of both 5-LOX and FLAP. Other studies demonstrated, however, that the increase in LTB_4 production was related mainly to up-regulation of the FLAP gene (Peters-Golden and Brock, 2003) or to a combination with an increase in the activity and/or expression of 5-LOX (Steinhilber et al., 1993;Brungs et al., 1995).

Role of PGE_2 and TGF-β1 in 5-LOX and FLAP expression

PGE_2 does not only participate in inflammation by itself, but may also regulate the effects of other inflammatory mediators, resulting in greater inflammatory and immune responses. Several studies reported that eicosanoid products of AA metabolism are important modulators of COX and 5-LOX pathways in various types of cells. For example, 5-LOX activity can be inhibited by some metabolites of the LOX pathway. PGE_2 can inhibit the synthesis of LTB_4 in rat neutrophils and chondrocytes (Ham et al., 1983). Another study showed that the expression of mPGES-1 is regulated by PGE_2 that may act as an enhancer of mPGES-1 in IL-1β stimulated rheumatoid synovial fibroblasts (Kojima et al., 2003). All these findings help us to better understand how PGE_2 and LTB_4 induced by HNE modulate various cell responses and AA metabolism in OA chondrocytes. Several studies showed that both FLAP and 5-LOX expressions were regulated by PGE_2 (Harizi et al., 2003;Martel-Pelletier et al., 2004). Our results put this observation in evidence. PGE_2 regulates FLAP and 5-LOX expression in different manners. The non-specific inhibitor of COXs naproxen abolishes HNE-induced

FLAP expression in OA chondrocytes after 24 and 72 h of incubation. In contrast, naproxen stimulates 5-LOX expression compared to unstimulated cells. We observed that 5-LOX is expressed only after 72 h of HNE incubation when the level of PGE_2 was low. This suggests that PGE_2 stimulates FLAP expression but inhibits 5-LOX expression. Important advances have been made concerning the role of PGE_2 in metabolism of articular tissues. It was found to be involved not only in inflammation responses but may regulate the effects of other inflammatory mediators. Therefore, more pharmaceutical companies put their interest in mPGES-1, the protein synthesizing PGE_2, to treat arthritis due to its inducible form and functional link with COX-2 (Fahmi, 2004). In contrast, PGE_2 inhibits 5-LOX after long stimulation time where 5-LOX just starts to be expressed. Our results are different than what the group of Harizi had obtained. They observed that PGE_2 reduces LTB_4 release by inhibiting FLAP via IL-10 dependent mechanism in dendritic cells, but without any effect on 5-LOX (Harizi et al., 2003). This suggests that PGE_2 regulates the expression of FLAP and 5-LOX differently depending on the types of cells.

The actual therapies to treat OA are limited to reducing the pain in affected joints by using either non-selective NSAID or the selective COX-2 drugs. Production of both PGE_2 and LTB_4 requires the existence of arachidonic acid, but their synthesis is through different pathways, COX and 5-LOX pathway respectively, therefore one of the pathways is favorably to be used depending on the condition. Long-term inhibition of COX causes the switch from COX to 5-

LOX pathway. This is observed in OA patients treated with COX-2 inhibitors (Maxis et al., 2006). In our study, we also demonstrated that lower PGE_2 production favors 5-LOX pathway in HNE-treated OA chondrocytes, leading to the synthesis of LTB_4. However, LTs have greater potential in inflammation process and may be more harmful than PGE_2 in various tissues due to their chemotactic property (Griffiths et al., 1995). For this reason, drugs that can inhibit both COX and LOX attract pharmaceutical attention in the treatment of inflammatory diseases. Tepoxalin and licofelone (ML3000) are drugs identified to have this function (Knight et al., 1996;Paredes et al., 2002). Today, licofelone is in phase III clinical development. It does not only lower the production of PGE_2 and LTB_4 but also can modify abnormal bone remodeling in OA; thus it was thought to be useful to treat OA (Paredes et al., 2002).

In addition to the involvement of PGE_2 in HNE-induced 5-LOX/FLAP, we found additional data supporting the role of TGF-β1 in this process. Our findings showed that HNE induced TGF-β1 and that TGF-β1 neutralization by using anti-TGF-β1 antibody attenuates HNE-induced 5-LOX and FLAP after 72 h of incubation. These results suggest that the regulation of 5-LOX/FLAP by HNE is mediated partially via TGF-β1 production. Leonarduzzi et al. 1997 were the first to demonstrate that HNE induced TGF-β1 production. Furthermore, there are a number of reports supporting the role of TGF-β1 in 5-LOX and FLAP expression through activation of smad 3/4 (Brungs et al., 1995;Martel-Pelletier et al.,

2004;Seuter et al., 2006). All these findings suggest that HNE stimulates the production of TGF-β1, which probably induces the expression of 5-LOX and FLAP through smad 3/4 involvement.

CHAPTER V: CONCLUSION

High levels of ROS and HNE are present in OA synovial tissues. Our study has several important implications and suggests that HNE production in OA articular tissues contributes to inflammatory responses in OA. We investigated the effect of HNE on the production of inflammatory mediators PGE_2 and LTB_4 and on the expression of COX-2, mPGES-1 and FLAP/5-LOX, the primary enzymes responsible for their biosynthesis. HNE induces differently the production of PGE_2 and LTB_4. Depletion of PGE_2 promotes LTB_4 formation, leading to the switch of AA metabolism from COX to FLAP/5-LOX pathway. The expression of FLAP and mainly 5-LOX increase after HNE stimulation when that of COX-2 and mPGES-1 decrease. In addition, the expression of FLAP happens earlier than that 5-LOX, which induces late biosynthesis of LTB_4. Furthermore, PGE_2 and TGF-β1 have regulatory effect on the expression of FLAP and 5-LOX at mRNA level.

A lot of perspectives are given by this study. Activations of certain transcriptional factors involved in the regulation of mPGES-1 and 5-LOX by HNE remain to be investigated, such as smad 2/3 and VDR for mPGES-1 and 5-LOX promoter studies. This will help us to better understand the role of smad 2/3 and VDR in mPGES-1 and 5-LOX expression. Also, it will be interesting to study the mechanism underlying the switch of pathways in AA metabolism.

Pathogenesis of OA is mainly characterized by inflammatory and catabolic process in parallel with high presence of several mediators, including ROS, HNE,

NO and PGE_2. We gave evidence that HNE stimulates the production of pro-inflammatory mediators PGE_2 and LTB_4 in human OA chondrocytes. Importantly, these factors do not only provoke inflammatory response but may also be involved in cartilage destruction. High concentration of PGE_2 can inhibit collagen synthesis in differentiated osteoblasts and LTB_4 has been identified to be a potent factor that induces TNF-α, IL-1β and MMPs. In a pharmaceutical point of view, inhibition of both the prostaglandins and LTs biosynthesis pathway is better than single interference due to the additive anti-inflammatory effectiveness and lower gastrointestinal toxicity. Therefore, a drug that has this effect may become an innovative treatment of OA and other inflammatory diseases. This study helps us to better understand the regulation of inflammatory factors and primary enzymes involved in OA by HNE.

CHAPTER VI: REFERENCES

Reference List

Ackerman,W.E., Summerfield,T.L., Vandre,D.D., Robinson,J.M., and Kniss,D.A. 2008. Nuclear factor-kappa B regulates inducible prostaglandin E synthase expression in human amnion mesenchymal cells. *Biol. Reprod.* 78:68-76.

Afif,H., Benderdour,M., Mfuna-Endam,L., Martel-Pelletier,J., Pelletier,J.P., Duval,N., and Fahmi,H. 2007. Peroxisome proliferator-activated receptor gamma1 expression is diminished in human osteoarthritic cartilage and is downregulated by interleukin-1beta in articular chondrocytes. *Arthritis Res. Ther.* 9:R31.

Aigner,T., and McKenna,L. 2002. Molecular pathology and pathobiology of osteoarthritic cartilage. *Cell Mol. Life Sci.* 59:5-18.

Aldini,G., Granata,P., and Carini,M. 2002. Detoxification of cytotoxic alpha,beta-unsaturated aldehydes by carnosine: characterization of conjugated adducts by electrospray ionization tandem mass spectrometry and detection by liquid chromatography/mass spectrometry in rat skeletal muscle. *J. Mass Spectrom.* 37:1219-1228.

Altman,R., Asch,E., Bloch,D., Bole,G., Borenstein,D., Brandt,K., Christy,W., Cooke,T.D., Greenwald,R., Hochberg,M., and al. 1986. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria

Committee of the American Rheumatism Association. *Arthritis Rheum.* 29:1039-1049.

Amin,A.R., Attur,M., Patel,R.N., Thakker,G.D., Marshall,P.J., Rediske,J., Stuchin,S.A., Patel,I.R., and Abramson,S.B. 1997. Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage. Influence of nitric oxide. *J. Clin. Invest* 99:1231-1237.

Aparoy,P., Reddy,R.N., Guruprasad,L., Reddy,M.R., and Reddanna,P. 2008. Homology modeling of 5-lipoxygenase and hints for better inhibitor design. *J. Comput. Aided Mol. Des* 22:611-619.

Arend,W.P., and Dayer,J.M. 1995. Inhibition of the production and effects of interleukin-1 and tumor necrosis factor alpha in rheumatoid arthritis. *Arthritis Rheum.* 38:151-160.

Babizhayev,M.A., Seguin,M.C., Gueyne,J., Evstigneeva,R.P., Ageyeva,E.A., and Zheltukhina,G.A. 1994. L-carnosine (beta-alanyl-L-histidine) and carcinine (beta-alanylhistamine) act as natural antioxidants with hydroxyl-radical-scavenging and lipid-peroxidase activities. *Biochem. J.* 304 (Pt 2):509-516.

Badger,A.M., Cook,M.N., Lark,M.W., Newman-Tarr,T.M., Swift,B.A., Nelson,A.H., Barone,F.C., and Kumar,S. 1998. SB 203580 inhibits p38 mitogen-activated protein kinase, nitric oxide production, and inducible

nitric oxide synthase in bovine cartilage-derived chondrocytes. *J. Immunol.* 161:467-473.

Benderdour,M., Tardif,G., Pelletier,J.P., Di Battista,J.A., Reboul,P., Ranger,P., and Martel-Pelletier,J. 2002. Interleukin 17 (IL-17) induces collagenase-3 production in human osteoarthritic chondrocytes via AP-1 dependent activation: differential activation of AP-1 members by IL-17 and IL-1beta. *J. Rheumatol.* 29:1262-1272.

Bergink,A.P., Uitterlinden,A.G., Van Leeuwen,J.P., Buurman,C.J., Hofman,A., Verhaar,J.A., and Pols,H.A. 2009. Vitamin D status, bone mineral density, and the development of radiographic osteoarthritis of the knee: The Rotterdam Study. *J. Clin. Rheumatol.* 15:230-237.

Bhosale,A.M., and Richardson,J.B. 2008. Articular cartilage: structure, injuries and review of management. *Br. Med. Bull.* 87:77-95.

Bigby,T.D. 1999. Regulation of expression of the 5-lipoxygenase pathway. *Clin. Rev. Allergy Immunol.* 17:43-58.

Blanc,E.M., Keller,J.N., Fernandez,S., and Mattson,M.P. 1998. 4-hydroxynonenal, a lipid peroxidation product, impairs glutamate transport in cortical astrocytes. *Glia* 22:149-160.

Brungs,M., Radmark,O., Samuelsson,B., and Steinhilber,D. 1994. On the induction of 5-lipoxygenase expression and activity in HL-60 cells: effects

of vitamin D3, retinoic acid, DMSO and TGF beta. *Biochem. Biophys. Res. Commun.* 205:1572-1580.

Brungs,M., Radmark,O., Samuelsson,B., and Steinhilber,D. 1995. Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor beta and 1,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. U. S. A* 92:107-111.

Buckwalter,J.A., and Lane,N.E. 1997a. Athletics and osteoarthritis. *Am. J. Sports Med.* 25:873-881.

Buckwalter,J.A., and Lane,N.E. 1997b. Does participation in sports cause osteoarthritis? *Iowa Orthop. J.* 17:80-89.

Catala,A. 2009. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chem. Phys. Lipids* 157:1-11.

Cerbone,A., Toaldo,C., Laurora,S., Briatore,F., Pizzimenti,S., Dianzani,M.U., Ferretti,C., and Barrera,G. 2007. 4-Hydroxynonenal and PPARgamma ligands affect proliferation, differentiation, and apoptosis in colon cancer cells. *Free Radic. Biol. Med.* 42:1661-1670.

Chen,A.F., Davies,C.M., De,L.M., and Fermor,B. 2008. Oxidative DNA damage in osteoarthritic porcine articular cartilage. *J. Cell Physiol* 217:828-833.

Cheng,S., Afif,H., Martel-Pelletier,J., Pelletier,J.P., Li,X., Farrajota,K., Lavigne,M., and Fahmi,H. 2004. Activation of peroxisome proliferator-activated receptor gamma inhibits interleukin-1beta-induced membrane-associated prostaglandin E2 synthase-1 expression in human synovial fibroblasts by interfering with Egr-1. *J. Biol. Chem.* 279:22057-22065.

Chu,S.J., Tang,L.O., Watney,E., Chi,E.Y., and Henderson,W.R., Jr. 2000. In situ amplification of 5-lipoxygenase and 5-lipoxygenase-activating protein in allergic airway inflammation and inhibition by leukotriene blockade. *J. Immunol.* 165:4640-4648.

Coffey,M.J., Wilcoxen,S.E., and Peters-Golden,M. 1994. Increases in 5-lipoxygenase activating protein expression account for enhanced capacity for 5-lipoxygenase metabolism that accompanies differentiation of peripheral blood monocytes into alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 11:153-158.

Comporti,M. 1985. Lipid peroxidation and cellular damage in toxic liver injury. *Lab Invest* 53:599-623.

Davies,C.M., Guilak,F., Weinberg,J.B., and Fermor,B. 2008. Reactive nitrogen and oxygen species in interleukin-1-mediated DNA damage associated with osteoarthritis. *Osteoarthritis. Cartilage.* 16:624-630.

Dieppe,P., Chard,J., Lohmander,S., and Smith,C. 2002. Osteoarthritis. *Clin. Evid.* 1071-1090.

Esterbauer,H., Schaur,R.J., and Zollner,H. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* 11:81-128.

Fahmi,H. 2004. mPGES-1 as a novel target for arthritis. *Curr. Opin. Rheumatol.* 16:623-627.

Felson,D.T. 1990. The epidemiology of knee osteoarthritis: results from the Framingham Osteoarthritis Study. *Semin. Arthritis Rheum.* 20:42-50.

Felson,D.T., Lawrence,R.C., Dieppe,P.A., Hirsch,R., Helmick,C.G., Jordan,J.M., Kington,R.S., Lane,N.E., Nevitt,M.C., Zhang,Y., Sowers,M., McAlindon,T., Spector,T.D., Poole,A.R., Yanovski,S.Z., Ateshian,G., Sharma,L., Buckwalter,J.A., Brandt,K.D., and Fries,J.F. 2000. Osteoarthritis: new insights. Part 1: the disease and its risk factors. *Ann. Intern. Med.* 133:635-646.

Felson,D.T., and Zhang,Y. 1998. An update on the epidemiology of knee and hip osteoarthritis with a view to prevention. *Arthritis Rheum.* 41:1343-1355.

Felson,D.T., Zhang,Y., Hannan,M.T., Naimark,A., Weissman,B.N., Aliabadi,P., and Levy,D. 1995. The incidence and natural history of knee osteoarthritis

in the elderly. The Framingham Osteoarthritis Study. *Arthritis Rheum.* 38:1500-1505.

Fernandes,J.C., Martel-Pelletier,J., and Pelletier,J.P. 2002. The role of cytokines in osteoarthritis pathophysiology. *Biorheology* 39:237-246.

FitzGerald,G.A. 2003. COX-2 and beyond: Approaches to prostaglandin inhibition in human disease. *Nat. Rev. Drug Discov.* 2:879-890.

Fu,M., Zhang,J., Lin,Y., Zhu,X., Ehrengruber,M.U., and Chen,Y.E. 2002. Early growth response factor-1 is a critical transcriptional mediator of peroxisome proliferator-activated receptor-gamma 1 gene expression in human aortic smooth muscle cells. *J. Biol. Chem.* 277:26808-26814.

Gabay,O., Hall,D.J., Berenbaum,F., Henrotin,Y., and Sanchez,C. 2008. Osteoarthritis and obesity: experimental models. *Joint Bone Spine* 75:675-679.

Gardner,D.L. 1983. The nature and causes of osteoarthrosis. *Br. Med. J. (Clin. Res. Ed)* 286:418-424.

Gibson,J.S., McCartney,D., Sumpter,J., Fairfax,T.P., Milner,P.I., Edwards,H.L., and Wilkins,R.J. 2009. Rapid effects of hypoxia on H+ homeostasis in articular chondrocytes. *Pflugers Arch.* 458:1085-1092.

Griffiths,R.J., Pettipher,E.R., Koch,K., Farrell,C.A., Breslow,R., Conklyn,M.J., Smith,M.A., Hackman,B.C., Wimberly,D.J., Milici,A.J., and al. 1995. Leukotriene B4 plays a critical role in the progression of collagen-induced arthritis. *Proc. Natl. Acad. Sci. U. S. A* 92:517-521.

Grigolo,B., Roseti,L., Fiorini,M., and Facchini,A. 2003. Enhanced lipid peroxidation in synoviocytes from patients with osteoarthritis. *J. Rheumatol.* 30:345-347.

Guerne,P.A., Zuraw,B.L., Vaughan,J.H., Carson,D.A., and Lotz,M. 1989. Synovium as a source of interleukin 6 in vitro. Contribution to local and systemic manifestations of arthritis. *J. Clin. Invest* 83:585-592.

Halliwell,B., and Chirico,S. 1993. Lipid peroxidation: its mechanism, measurement, and significance. *Am. J. Clin. Nutr.* 57:715S-724S.

Ham,E.A., Soderman,D.D., Zanetti,M.E., Dougherty,H.W., McCauley,E., and Kuehl,F.A., Jr. 1983. Inhibition by prostaglandins of leukotriene B4 release from activated neutrophils. *Proc. Natl. Acad. Sci. U. S. A* 80:4349-4353.

Han,R., Tsui,S., and Smith,T.J. 2002. Up-regulation of prostaglandin E2 synthesis by interleukin-1beta in human orbital fibroblasts involves coordinate induction of prostaglandin-endoperoxide H synthase-2 and glutathione-dependent prostaglandin E2 synthase expression. *J. Biol. Chem.* 277:16355-16364.

Haq,I., Murphy,E., and Dacre,J. 2003. Osteoarthritis. *Postgrad. Med. J.* 79:377-383.

Hardy,M.M., Seibert,K., Manning,P.T., Currie,M.G., Woerner,B.M., Edwards,D., Koki,A., and Tripp,C.S. 2002. Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. *Arthritis Rheum.* 46:1789-1803.

Harizi,H., Juzan,M., Moreau,J.F., and Gualde,N. 2003. Prostaglandins inhibit 5-lipoxygenase-activating protein expression and leukotriene B4 production from dendritic cells via an IL-10-dependent mechanism. *J. Immunol.* 170:139-146.

Harle,D., Radmark,O., Samuelsson,B., and Steinhilber,D. 1999. Transcriptional and posttranscriptional regulation of 5-lipoxygenase mRNA expression in the human monocytic cell line Mono Mac 6 by transforming growth factor-beta and 1,25-dihydroxyvitamin D3. *Adv. Exp. Med. Biol.* 469:105-111.

Hart,P.H., Vitti,G.F., Burgess,D.R., Whitty,G.A., Piccoli,D.S., and Hamilton,J.A. 1989. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. *Proc. Natl. Acad. Sci. U. S. A* 86:3803-3807.

Hasan,R.N., and Schafer,A.I. 2008. Hemin upregulates Egr-1 expression in vascular smooth muscle cells via reactive oxygen species ERK-1/2-Elk-1 and NF-kappaB. *Circ. Res.* 102:42-50.

Henrotin,Y.E., Bruckner,P., and Pujol,J.P. 2003. The role of reactive oxygen species in homeostasis and degradation of cartilage. *Osteoarthritis. Cartilage.* 11:747-755.

Hunter,D.J., March,L., and Sambrook,P.N. 2002. Knee osteoarthritis: the influence of environmental factors. *Clin. Exp. Rheumatol.* 20:93-100.

Joosten,L.A., Lubberts,E., Durez,P., Helsen,M.M., Jacobs,M.J., Goldman,M., and Van den Berg,W.B. 1997. Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. *Arthritis Rheum.* 40:249-260.

Jovanovic,D., Pelletier,J.P., Alaaeddine,N., Mineau,F., Geng,C., Ranger,P., and Martel-Pelletier,J. 1998. Effect of IL-13 on cytokines, cytokine receptors and inhibitors on human osteoarthritis synovium and synovial fibroblasts. *Osteoarthritis. Cartilage.* 6:40-49.

Kerin,A., Patwari,P., Kuettner,K., Cole,A., and Grodzinsky,A. 2002. Molecular basis of osteoarthritis: biomechanical aspects. *Cell Mol. Life Sci.* 59:27-35.

Knight,E.V., Kimball,J.P., Keenan,C.M., Smith,I.L., Wong,F.A., Barrett,D.S., Dempster,A.M., Lieuallen,W.G., Panigrahi,D., Powers,W.J., and Szot,R.J. 1996. Preclinical toxicity evaluation of tepoxalin, a dual inhibitor of cyclooxygenase and 5-lipoxygenase, in Sprague-Dawley rats and beagle dogs. *Fundam. Appl. Toxicol.* 33:38-48.

Kojima,F., Naraba,H., Miyamoto,S., Beppu,M., Aoki,H., and Kawai,S. 2004. Membrane-associated prostaglandin E synthase-1 is upregulated by proinflammatory cytokines in chondrocytes from patients with osteoarthritis. *Arthritis Res. Ther.* 6:R355-R365.

Kojima,F., Naraba,H., Sasaki,Y., Beppu,M., Aoki,H., and Kawai,S. 2003. Prostaglandin E2 is an enhancer of interleukin-1beta-induced expression of membrane-associated prostaglandin E synthase in rheumatoid synovial fibroblasts. *Arthritis Rheum.* 48:2819-2828.

Kowal-Bielecka,O., Distler,O., Neidhart,M., Kunzler,P., Rethage,J., Nawrath,M., Carossino,A., Pap,T., Muller-Ladner,U., Michel,B.A., Sierakowski,S., Matucci-Cerinic,M., Gay,R.E., and Gay,S. 2001. Evidence of 5-lipoxygenase overexpression in the skin of patients with systemic sclerosis: a newly identified pathway to skin inflammation in systemic sclerosis. *Arthritis Rheum.* 44:1865-1875.

Krump,E., and Borgeat,P. 1994. Kinetics of 5-lipoxygenase activation, arachidonic acid release, and leukotriene synthesis in human neutrophils: effects of

granulocyte-macrophage colony-stimulating factor. *Biochim. Biophys. Acta* 1213:135-139.

Kumagai,T., Kawamoto,Y., Nakamura,Y., Hatayama,I., Satoh,K., Osawa,T., and Uchida,K. 2000. 4-hydroxy-2-nonenal, the end product of lipid peroxidation, is a specific inducer of cyclooxygenase-2 gene expression. *Biochem. Biophys. Res. Commun.* 273:437-441.

Kumagai,T., Matsukawa,N., Kaneko,Y., Kusumi,Y., Mitsumata,M., and Uchida,K. 2004. A lipid peroxidation-derived inflammatory mediator: identification of 4-hydroxy-2-nonenal as a potential inducer of cyclooxygenase-2 in macrophages. *J. Biol. Chem.* 279:48389-48396.

Kumagai,T., Nakamura,Y., Osawa,T., and Uchida,K. 2002. Role of p38 mitogen-activated protein kinase in the 4-hydroxy-2-nonenal-induced cyclooxygenase-2 expression. *Arch. Biochem. Biophys.* 397:240-245.

Lascelles,B.D., King,S., Roe,S., Marcellin-Little,D.J., and Jones,S. 2009. Expression and activity of COX-1 and 2 and 5-LOX in joint tissues from dogs with naturally occurring coxofemoral joint osteoarthritis. *J. Orthop. Res.* 27:1204-1208.

Lee,S.J., Kim,C.E., Yun,M.R., Seo,K.W., Park,H.M., Yun,J.W., Shin,H.K., Bae,S.S., and Kim,C.D. 2010. 4-Hydroxynonenal enhances MMP-9

production in murine macrophages via 5-lipoxygenase-mediated activation of ERK and p38 MAPK. *Toxicol. Appl. Pharmacol.* 242:191-198.

LeGrand,A., Fermor,B., Fink,C., Pisetsky,D.S., Weinberg,J.B., Vail,T.P., and Guilak,F. 2001. Interleukin-1, tumor necrosis factor alpha, and interleukin-17 synergistically up-regulate nitric oxide and prostaglandin E2 production in explants of human osteoarthritic knee menisci. *Arthritis Rheum.* 44:2078-2083.

Leonarduzzi,G., Scavazza,A., Biasi,F., Chiarpotto,E., Camandola,S., Vogel,S., Dargel,R., and Poli,G. 1997. The lipid peroxidation end product 4-hydroxy-2,3-nonenal up-regulates transforming growth factor beta1 expression in the macrophage lineage: a link between oxidative injury and fibrosclerosis. *FASEB J.* 11:851-857.

Liu,W., Kato,M., Akhand,A.A., Hayakawa,A., Suzuki,H., Miyata,T., Kurokawa,K., Hotta,Y., Ishikawa,N., and Nakashima,I. 2000. 4-hydroxynonenal induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death. *J. Cell Sci.* 113 (Pt 4):635-641.

Martel-Pelletier,J. 2004. Pathophysiology of osteoarthritis. *Osteoarthritis. Cartilage.* 12 Suppl A:S31-S33.

Martel-Pelletier,J., Lajeunesse,D., Reboul,P., and Pelletier,J.P. 2003. Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. *Ann. Rheum. Dis.* 62:501-509.

Martel-Pelletier,J., Mineau,F., Fahmi,H., Laufer,S., Reboul,P., Boileau,C., Lavigne,M., and Pelletier,J.P. 2004. Regulation of the expression of 5-lipoxygenase-activating protein/5-lipoxygenase and the synthesis of leukotriene B(4) in osteoarthritic chondrocytes: role of transforming growth factor beta and eicosanoids. *Arthritis Rheum.* 50:3925-3933.

Martel-Pelletier,J., Welsch,D.J., and Pelletier,J.P. 2001. Metalloproteases and inhibitors in arthritic diseases. *Best. Pract. Res. Clin. Rheumatol.* 15:805-829.

Mattson,M.P. 2009. Roles of the lipid peroxidation product 4-hydroxynonenal in obesity, the metabolic syndrome, and associated vascular and neurodegenerative disorders. *Exp. Gerontol.* 44:625-633.

Maxis,K., Delalandre,A., Martel-Pelletier,J., Pelletier,J.P., Duval,N., and Lajeunesse,D. 2006. The shunt from the cyclooxygenase to lipoxygenase pathway in human osteoarthritic subchondral osteoblasts is linked with a variable expression of the 5-lipoxygenase-activating protein. *Arthritis Res. Ther.* 8:R181.

Milner,P.I., Wilkins,R.J., and Gibson,J.S. 2007. The role of mitochondrial reactive oxygen species in pH regulation in articular chondrocytes. *Osteoarthritis. Cartilage.* 15:735-742.

Moilanen,M., Sorsa,T., Stenman,M., Nyberg,P., Lindy,O., Vesterinen,J., Paju,A., Konttinen,Y.T., Stenman,U.H., and Salo,T. 2003. Tumor-associated trypsinogen-2 (trypsinogen-2) activates procollagenases (MMP-1, -8, -13) and stromelysin-1 (MMP-3) and degrades type I collagen. *Biochemistry* 42:5414-5420.

Morquette,B., Shi,Q., Lavigne,P., Ranger,P., Fernandes,J.C., and Benderdour,M. 2006. Production of lipid peroxidation products in osteoarthritic tissues: new evidence linking 4-hydroxynonenal to cartilage degradation. *Arthritis Rheum.* 54:271-281.

Murphy,R.C., and Gijon,M.A. 2007. Biosynthesis and metabolism of leukotrienes. *Biochem. J.* 405:379-395.

Nagase,H., and Kashiwagi,M. 2003. Aggrecanases and cartilage matrix degradation. *Arthritis Res. Ther.* 5:94-103.

Naraba,H., Yokoyama,C., Tago,N., Murakami,M., Kudo,I., Fueki,M., Oh-Ishi,S., and Tanabe,T. 2002. Transcriptional regulation of the membrane-associated prostaglandin E2 synthase gene. Essential role of the transcription factor Egr-1. *J. Biol. Chem.* 277:28601-28608.

Nevitt,M.C., Felson,D.T., Williams,E.N., and Grady,D. 2001. The effect of estrogen plus progestin on knee symptoms and related disability in postmenopausal women: The Heart and Estrogen/Progestin Replacement Study, a randomized, double-blind, placebo-controlled trial. *Arthritis Rheum.* 44:811-818.

Noguchi,M., Miyano,M., and Matsumoto,T. 1996. Physiochemical characterization of ATP binding to human 5-lipoxygenase. *Lipids* 31:367-371.

Okada,K., Wangpoengtrakul,C., Osawa,T., Toyokuni,S., Tanaka,K., and Uchida,K. 1999. 4-Hydroxy-2-nonenal-mediated impairment of intracellular proteolysis during oxidative stress. Identification of proteasomes as target molecules. *J. Biol. Chem.* 274:23787-23793.

Oliveria,S.A., Felson,D.T., Reed,J.I., Cirillo,P.A., and Walker,A.M. 1995. Incidence of symptomatic hand, hip, and knee osteoarthritis among patients in a health maintenance organization. *Arthritis Rheum.* 38:1134-1141.

Ouedraogo,D.D., Seogo,H., Cisse,R., Tieno,H., Ouedraogo,T., Nacoulma,I.S., and Drabo,Y.J. 2008. [Risk factors associated with osteoarthritis of the knee in a rheumatology outpatient clinic in Ouagadougou, Burkina Faso]. *Med. Trop. (Mars.)* 68:597-599.

Pappa,A., Estey,T., Manzer,R., Brown,D., and Vasiliou,V. 2003. Human aldehyde dehydrogenase 3A1 (ALDH3A1): biochemical characterization and immunohistochemical localization in the cornea. *Biochem. J.* 376:615-623.

Paredes,Y., Massicotte,F., Pelletier,J.P., Martel-Pelletier,J., Laufer,S., and Lajeunesse,D. 2002. Study of the role of leukotriene B4 in abnormal function of human subchondral osteoarthritis osteoblasts: effects of cyclooxygenase and/or 5-lipoxygenase inhibition. *Arthritis Rheum.* 46:1804-1812.

Park,J.Y., Pillinger,M.H., and Abramson,S.B. 2006. Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases. *Clin. Immunol.* 119:229-240.

Partridge,R.E., and Duthie,J.J. 1968. Rheumatism in dockers and civil servants. A comparison of heavy manual and sedentary workers. *Ann. Rheum. Dis.* 27:559-568.

Pelletier,J.P., DiBattista,J.A., Roughley,P., McCollum,R., and Martel-Pelletier,J. 1993. Cytokines and inflammation in cartilage degradation. *Rheum. Dis. Clin. North Am.* 19:545-568.

Peters-Golden,M. 1998. Cell biology of the 5-lipoxygenase pathway. *Am. J. Respir. Crit Care Med.* 157:S227-S232.

Peters-Golden,M., and Brock,T.G. 2003. 5-lipoxygenase and FLAP. *Prostaglandins Leukot. Essent. Fatty Acids* 69:99-109.

Pizzimenti,S., Laurora,S., Briatore,F., Ferretti,C., Dianzani,M.U., and Barrera,G. 2002. Synergistic effect of 4-hydroxynonenal and PPAR ligands in controlling human leukemic cell growth and differentiation. *Free Radic. Biol. Med.* 32:233-245.

Poli,G., Albano,E., and Dianzani,M.U. 1987. The role of lipid peroxidation in liver damage. *Chem. Phys. Lipids* 45:117-142.

Poli,G., Schaur,R.J., Siems,W.G., and Leonarduzzi,G. 2008. 4-hydroxynonenal: a membrane lipid oxidation product of medicinal interest. *Med. Res. Rev.* 28:569-631.

Poole,A.R. 1999. An introduction to the pathophysiology of osteoarthritis. *Front Biosci.* 4:D662-D670.

Poole,A.R., Kojima,T., Yasuda,T., Mwale,F., Kobayashi,M., and Laverty,S. 2001. Composition and structure of articular cartilage: a template for tissue repair. *Clin. Orthop. Relat Res.* S26-S33.

Quinn,P.J., Boldyrev,A.A., and Formazuyk,V.E. 1992. Carnosine: its properties, functions and potential therapeutic applications. *Mol. Aspects Med.* 13:379-444.

Radin,E.L., Burr,D.B., Caterson,B., Fyhrie,D., Brown,T.D., and Boyd,R.D. 1991. Mechanical determinants of osteoarthrosis. *Semin. Arthritis Rheum.* 21:12-21.

Reboul,P., Pelletier,J.P., Tardif,G., Cloutier,J.M., and Martel-Pelletier,J. 1996. The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis. *J. Clin. Invest* 97:2011-2019.

Roman-Blas,J.A., Contreras-Blasco,M.A., Largo,R., Alvarez-Soria,M.A., Castaneda,S., and Herrero-Beaumont,G. 2009. Differential effects of the antioxidant n-acetylcysteine on the production of catabolic mediators in IL-1beta-stimulated human osteoarthritic synoviocytes and chondrocytes. *Eur. J. Pharmacol.* 623:125-131.

Rouzer,C.A., and Samuelsson,B. 1987. Reversible, calcium-dependent membrane association of human leukocyte 5-lipoxygenase. *Proc. Natl. Acad. Sci. U. S. A* 84:7393-7397.

Sandell,L.J., and Aigner,T. 2001. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res.* 3:107-113.

Schmidt,T.A., Gastelum,N.S., Nguyen,Q.T., Schumacher,B.L., and Sah,R.L. 2007. Boundary lubrication of articular cartilage: role of synovial fluid constituents. *Arthritis Rheum.* 56:882-891.

Schroder,J.M. 1989. The monocyte-derived neutrophil activating peptide (NAP/interleukin 8) stimulates human neutrophil arachidonate-5-

lipoxygenase, but not the release of cellular arachidonate. *J. Exp. Med.* 170:847-863.

Seo,K.W., Lee,S.J., Kim,C.E., Yun,M.R., Park,H.M., Yun,J.W., Bae,S.S., and Kim,C.D. 2010. Participation of 5-lipoxygenase-derived LTB(4) in 4-hydroxynonenal-enhanced MMP-2 production in vascular smooth muscle cells. *Atherosclerosis* 208:56-61.

Seuter,S., Sorg,B.L., and Steinhilber,D. 2006. The coding sequence mediates induction of 5-lipoxygenase expression by Smads3/4. *Biochem. Biophys. Res. Commun.* 348:1403-1410.

Shi,Q., Vaillancourt,F., Cote,V., Fahmi,H., Lavigne,P., Afif,H., Di Battista,J.A., Fernandes,J.C., and Benderdour,M. 2006. Alterations of metabolic activity in human osteoarthritic osteoblasts by lipid peroxidation end product 4-hydroxynonenal. *Arthritis Res. Ther.* 8:R159.

Siegle,I., Klein,T., Backman,J.T., Saal,J.G., Nusing,R.M., and Fritz,P. 1998. Expression of cyclooxygenase 1 and cyclooxygenase 2 in human synovial tissue: differential elevation of cyclooxygenase 2 in inflammatory joint diseases. *Arthritis Rheum.* 41:122-129.

Siems,W., and Grune,T. 2003. Intracellular metabolism of 4-hydroxynonenal. *Mol. Aspects Med.* 24:167-175.

Siems,W.G., Brenke,R., Beier,A., and Grune,T. 2002. Oxidative stress in chronic lymphoedema. *QJM.* 95:803-809.

Silverman,E.S., Du,J., De Sanctis,G.T., Radmark,O., Samuelsson,B., Drazen,J.M., and Collins,T. 1998. Egr-1 and Sp1 interact functionally with the 5-lipoxygenase promoter and its naturally occurring mutants. *Am. J. Respir. Cell Mol. Biol.* 19:316-323.

Smith,W.L., DeWitt,D.L., and Garavito,R.M. 2000. Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.* 69:145-182.

Stadler,J., Stefanovic-Racic,M., Billiar,T.R., Curran,R.D., McIntyre,L.A., Georgescu,H.I., Simmons,R.L., and Evans,C.H. 1991. Articular chondrocytes synthesize nitric oxide in response to cytokines and lipopolysaccharide. *J. Immunol.* 147:3915-3920.

Steinhilber,D., Radmark,O., and Samuelsson,B. 1993. Transforming growth factor beta upregulates 5-lipoxygenase activity during myeloid cell maturation. *Proc. Natl. Acad. Sci. U. S. A* 90:5984-5988.

Stichtenoth,D.O., Thoren,S., Bian,H., Peters-Golden,M., Jakobsson,P.J., and Crofford,L.J. 2001. Microsomal prostaglandin E synthase is regulated by proinflammatory cytokines and glucocorticoids in primary rheumatoid synovial cells. *J. Immunol.* 167:469-474.

Subbaramaiah,K., Yoshimatsu,K., Scherl,E., Das,K.M., Glazier,K.D., Golijanin,D., Soslow,R.A., Tanabe,T., Naraba,H., and Dannenberg,A.J. 2004. Microsomal prostaglandin E synthase-1 is overexpressed in inflammatory bowel disease. Evidence for involvement of the transcription factor Egr-1. *J. Biol. Chem.* 279:12647-12658.

Szweda,L.I., Uchida,K., Tsai,L., and Stadtman,E.R. 1993. Inactivation of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. Selective modification of an active-site lysine. *J. Biol. Chem.* 268:3342-3347.

Tetlow,L.C., and Woolley,D.E. 2001. Expression of vitamin D receptors and matrix metalloproteinases in osteoarthritic cartilage and human articular chondrocytes in vitro. *Osteoarthritis. Cartilage.* 9:423-431.

Tsuchiya,Y., Yamaguchi,M., Chikuma,T., and Hojo,H. 2005. Degradation of glyceraldehyde-3-phosphate dehydrogenase triggered by 4-hydroxy-2-nonenal and 4-hydroxy-2-hexenal. *Arch. Biochem. Biophys.* 438:217-222.

Uchida,K. 2003. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Prog. Lipid Res.* 42:318-343.

Uchida,K. 2008. A lipid-derived endogenous inducer of COX-2: a bridge between inflammation and oxidative stress. *Mol. Cells* 25:347-351.

Uchida,K., and Stadtman,E.R. 1992a. Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proc. Natl. Acad. Sci. U. S. A* 89:4544-4548.

Uchida,K., and Stadtman,E.R. 1992b. Selective cleavage of thioether linkage in proteins modified with 4-hydroxynonenal. *Proc. Natl. Acad. Sci. U. S. A* 89:5611-5615.

Ullrich,O., Huser,H., Ehrlich,W., and Grune,T. 1997. Intracellular metabolism of 4-hydroxynonenal in primary cultures of rabbit synovial fibroblasts. *Free Radic. Biol. Med.* 22:1153-1157.

Uracz,W., Uracz,D., Olszanecki,R., and Gryglewski,R.J. 2002. Interleukin 1beta induces functional prostaglandin E synthase in cultured human umbilical vein endothelial cells. *J. Physiol Pharmacol.* 53:643-654.

Vaillancourt,F., Fahmi,H., Shi,Q., Lavigne,P., Ranger,P., Fernandes,J.C., and Benderdour,M. 2008. 4-Hydroxynonenal induces apoptosis in human osteoarthritic chondrocytes: the protective role of glutathione-S-transferase. *Arthritis Res. Ther.* 10:R107.

Vaillancourt,F., Morquette,B., Shi,Q., Fahmi,H., Lavigne,P., Di Battista,J.A., Fernandes,J.C., and Benderdour,M. 2007. Differential regulation of cyclooxygenase-2 and inducible nitric oxide synthase by 4-hydroxynonenal in human osteoarthritic chondrocytes through ATF-2/CREB-1

transactivation and concomitant inhibition of NF-kappaB signaling cascade. *J. Cell Biochem.* 100:1217-1231.

Van de Loo,F.A., Kuiper,S., van Enckevort,F.H., Arntz,O.J., and Van den Berg,W.B. 1997. Interleukin-6 reduces cartilage destruction during experimental arthritis. A study in interleukin-6-deficient mice. *Am. J. Pathol.* 151:177-191.

Wardlaw,A.J., Hay,H., Cromwell,O., Collins,J.V., and Kay,A.B. 1989. Leukotrienes, LTC4 and LTB4, in bronchoalveolar lavage in bronchial asthma and other respiratory diseases. *J. Allergy Clin. Immunol.* 84:19-26.

Westacott,C.I., and Sharif,M. 1996. Cytokines in osteoarthritis: mediators or markers of joint destruction? *Semin. Arthritis Rheum.* 25:254-272.

Yucel-Lindberg,T., Olsson,T., and Kawakami,T. 2006. Signal pathways involved in the regulation of prostaglandin E synthase-1 in human gingival fibroblasts. *Cell Signal.* 18:2131-2142.

Zarkovic,N. 2003. 4-hydroxynonenal as a bioactive marker of pathophysiological processes. *Mol. Aspects Med.* 24:281-291.