Université de Montréal

Selective inhibition of inducible nitric oxide synthase prevents lipid peroxidation in cartilage from patients with osteoarthritis.

L'inhibition sélective de l'oxyde nitrique synthétase inductible inhibe la péroxidation lipidique dans le cartilage humain arthrosique.

par

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Ce mémoire est intitulé :

L'inhibition sélective de l'oxyde nitrique synthétase inductible inhibe la péroxidation lipidique chez les patients souffrant de l'ostéoarthrose.

Présenté par : Mireille Bentz

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Dr Moutih Rafei, président-rapporteur

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Résumé

INTRODUCTION: De nouvelles données indiquent que l'oxyde nitrique (NO), que l'on retrouve en quantité accrue au niveau du cartilage de patients atteints d'ostéoarthrose (OA), joue un rôle dans la production de 4-hydrynonénal (HNE) via la formation de péroxynitrite ($ONOO^-$). Le HNE est considéré comme étant un des produits les plus réactifs de la péroxidation lipidique (LPO). Notre laboratoire a rapporté des niveaux de HNE plus élevés que normal dans le liquide synovial provenant de genoux de patients OA comparativement aux sujets normaux. Nous avions aussi démontré que le HNE peut induire la production des médiateurs inflammatoires et cataboliques connus pour leurs implications dans la dégradation du cartilage dans l'OA. Le but de la présente étude est de vérifier si un inhibiteur sélectif pour la NO synthétase inductible (iNOS), soit le L-NIL (L-N^6-(L-Iminoethyl)Lysine), peut empêcher la formation du HNE via l'inhibition de la production du NO dans des chondrocytes de patients OA.

MÉTHODES: Les cellules ont été traitées soit avec ou sans un générateur du NO (SIN ou interleukine-1beta (IL-1β)) soit avec ou sans du HNE pendant 48 h en présence ou en absence du L-NIL. L'expression protéique de l'iNOS et de la sous-unité de la NADPH oxydase (NOX), la p47 (phox), a été vérifiée par Western blot. La génération du HNE a été révélée par ELISA, Western blot et immunohistochimie. Les niveaux de prostaglandine E2 (PGE2), gluthation-s-transférase (GST) et de la MMP-13 ont été mesurés par des kits commerciaux. La quantité de NO a été évaluée par la méthode de la réaction de Griess. La mesure des niveaux d'espèces réactives d'oxygène (ROS) a été effectuée par fluorescence en utilisant un kit commercial.

RÉSULTATS: Le L-NIL inhibe la stimulation de la production de NO, de HNE ainsi que l'expression d'iNOS au niveau protéique et de l'ARNm par IL-1β. Le L-NIL bloque aussi la production de HNE indépendamment de la production de NO. La production des ROS et l'activation de p47 (phox) ont été inhibées par L-NIL. Fait intéressant, le L-NIL empêche la production de la PGE2, de la cyclooxygénase-2 (COX-2) et de la MMP-13 induite par le HNE.

CONCLUSION: les résultats obtenus semblent démontrer l'effet bénéfique du L-NIL dans l'OA via la prévention de la production du HNE de manière dépendante ou indépendant du NO.

MOTS CLÉS : Arthrose; Chondrocytes; Péroxydation Lipidique; 4-Hydroxynonénal; Oxyde Nitrique; Inflammation; Catabolisme; L-N6-(L-Iminoethyl)Lysine; Cartilage; Interleukine; Stress Oxydatif; Cible Thérapeutique; Oxyde Nitrique Synthétase Inductible; Espèces réactives d'Oxygène; Inhibition sélective

Abstract

INTRODUCTION: Emerging evidence indicates that nitric oxide (NO), which is increased in osteoarthritic (OA) cartilage, plays a role in 4-hydroxynonenal (HNE) generation through peroxynitrite formation. HNE is considered as the most reactive product of lipid peroxidation (LPO). We have previously reported that HNE levels in synovial fluids are more elevated in knees of OA patients compared to healthy individuals. We also demonstrated that HNE induces a panoply of inflammatory and catabolic mediators known for their implication in OA cartilage degradation. The aim of the present study was to investigate the ability of inducible NO synthase (iNOS) inhibitor, L-NIL (L-N6-(L-Iminoethyl)Lysine), to prevent HNE generation through NO inhibition in human OA chondrocytes.

METHOD: Cells and cartilage explants were treated with or without either an NO generator (SIN or interleukin 1beta (IL-1β)) or HNE in absence or presence of L-NIL. Protein expression of both iNOS and free-radical-generating NOX subunit p47 (phox) were investigated by western blot. iNOS mRNA detection was measured by real-time RT-PCR. HNE production was analysed by ELISA, Western blot and immunohistochemistry. S-nitrosylated proteins were evaluated by Western Blot. Prostaglandin E2 (PGE2) and metalloproteinase 13 (MMP-13) levels as well as glutathione S-transferase (GST) activity were each assessed with commercial kits. NO release was determined using improved Griess method. Reactive oxygen species (ROS) generation was revealed using fluorescent microscopy with the use of commercial kits.

RESULTS: L-NIL prevented IL-1β-induced NO release, iNOS expression at protein and mRNA levels, S-nitrosylated proteins and HNE in a dose dependent manner after 24h of incubation. Interestingly, we revealed that L-NIL abolished IL-1β-induced NOX component p47phox as well as ROS release. The HNE-induced PGE2 release and both cyclooxygenase-2 (COX-2) and MMP-13 expression were significantly reduced by L-NIL addition. Furthermore, L-NIL blocked the IL-1β induced inactivation of GST, an HNE-metabolizing enzyme. Also, L-NIL prevented HNE induced cell death at cytotoxic levels.

CONCLUSION: Altogether, our findings support a beneficial effect of L-NIL in OA by preventing LPO process in NO-dependent and/or independent mechanisms.

KEYWORDS : Osteoarthritis; Chondrocyte; Lipid peroxydation; 4-Hydroxynonenal; Catabolism; Inflammation; Nitric Oxide; L-N6-(L-Iminoethyl)Lysine; Cartilage; Interleukin; Oxydative Stress; Therapeutic target; Inducible Nitric Oxide Synthase; Reactive Oxygen Species; Selective Inhibition

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List of abbreviations

AA	Arachidonic Acid
ADAMTS	A Disintegrin And Metalloprotease With Thrombospondin Motifs
ADAMTS-4	Aggrecanase-1
ADAMTS-5	Aggrecanase-2
AP	Activator Protein
APAF-1	Apoptotic Protease Activating Factor-1
BMPs	Bone Morphogenetic Proteins
CC	Carbon-Carbon
CD40L	CD40 Ligand
CO	Carbonyl Group
COL2A1	Type II Collagen Gene A1
Coll II	Type 2 Collagen
COX	Cyclooxygenase
COX-1	Cyclooxygenase 1
COX2	Cyclooxygenase-2
CYS	Cysteine
CytoC	Cytochrome-C
DHN	1,4-Dihydroxynonene
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular Signal-Regulated Kinases
FasL	Fas Ligand
GSH	Glutathione
GST	Glutathione S-Transferase
H_2O_2	Hydrogen Peroxide
HIS	Histidine
HNA	Hydroxynonenoic Acid
HNE	4-Hydroxynonenal
Hsp-72	Heat Shock Proteins 72
Hsp-90	Heat Shock Proteins 90

ICE	Interleukin-1 Beta Converting Enzyme Or Caspase-1
IGF-I	Insulin-Like Growth Factor I
IKK	Inhibitors Of Kappa B Kinase
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-1R	Interleukin-1 Receptor
IL-1Ra	Interleukin-1 Receptor Antagonist
IL-1R-AcP	Interleukin-1 Receptor Accessory Protein
IL-1RI	Interleukin-1 Receptor Type I
IL-1RII	Interleukin-1 Receptor Type II
IL-1α	Interleukin-1 Alpha
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible Nitric Oxide Synthase
iNOS	Inducile Nitric Oxide Synthase
IκB	Inhibitor Of Keppa B
IκBα	Inhibitor Of Keppa B Alpha
IκBβ	Inhibitor Of Keppa B Beta
IκBγ	Inhibitor Of Keppa B Gamma
IκBε	Inhibitor Of Keppa B Epsilon
JNK	Jun NH_2-Terminal Kinase
JUNK1	Jun NH2-Terminal Kinase -1
L•	Lipid Radical
LH	Unsaturated Lipids
L-NIL	L-N^6-(L-Iminoethyl)Lysine
L-NMA	N^G-Monomethyl-L-Arginine
L-NNA	N^G-Nitro-L-Arginine
LOO•	Lipid Peroxyl Radical
LOOH	Hydroperoxides
LPO	Lipid Peroxidation

LTα	Lymphotoxin-Alpha
LYS	Lysine
MAPK	Mitogen-Activated Protein Kinases
MDA	Malondialdehyde
MMP-1	Matrix Metalloproteinase 1
MMP-13	Matrix Metalloproteinase 13
MMP-2	Matrix Metalloproteinase 2
MMP-3	Matrix Metalloproteinase 3
MMP-8	Matrix Metalloproteinase 8
MMP-9	Matrix Metalloproteinase 9
MMPs	Matrix Metalloproteinases
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFκB	Nuclear Factor Kappa B
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NOX	Nadph Oxidase
O_2	Oxygen
$O_2^{\bullet}$	Superoxide Anion
OA	Osteoarthritis
OH^-	Hydroxyl Radical
$ONOO^-$	Peroxynitrite
PG	Prostaglandin
PGE_2	Prostaglandin E2
PGG_2	Prostaglandins G2
PGHS	Prostaglandin H_2 Synthase
PLA_2	Phospholipase A_2
PUFAs	Polyunsaturated Fatty Acids
RA	Rheumatoid Arthritis
RANKL	Receptor-Activator Of Nuclear Factor Kappa B Ligand
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species

sIL-6R	Soluble Interleukin-6 Receptor
sTNRF-1	Soluble Tumor Necrosis Factor Receptor I
sTNRF-2	Soluble Tumor Necrosis Factor Receptor II
TGF-β	Transforming Growth Factor Beta
TIMP	Tissue Inhibitor Of Metalloproteins
TNF-RI	Tumour Necrosis Factor Alpha Receptor Type I
TNF-RII	Tumour Necrosis Factor Alpha Receptor Type II
TNF-α	Tumour Necrosis Factor Alpha
TX	Thromboxane
TYR	Tyrosine

Education is what remains after one

has forgotten what one has learned in school.

Albert Einstein

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CHAPITER 1: Introduction

1.1 Articular cartilage morphology

1.1.1 Structure and composition

Articular cartilage is made of hyaline cartilage and is found at bone extremities(Marieb 2005). It is both aneural and avascular, thus requiring the diffusion of nutrients contained within the synovial fluid as the main source of nourishment for its cells (Woessner Jr & Howell 1993; Eckstein et al. 2006). It is composed of an extracellular matrix (ECM), which is mostly composed of water (±70%), collagen (15%) and aggrecans (9%), as well as chondrocytes (3%), cartilage specific cells which are embedded in the ECM (see Figure 1)(Poole et al. 2001; Bollet & Nance 1966). The primary function of the articular cartilage is to provide a lubricated and even joint surface that enables smooth articulation (Poole et al. 2001). To do so, the cartilage cushions against compressive forces using hydrostatic pressurization while also protecting against tensile and shearing forces thanks to the presence of collagen(Marieb 2005; Eckstein et al. 2006; Poole et al. 2001). It thus protects the subchondral bone against abrasions and other stress-induced damages. Hyaline cartilage has a very low coefficient of friction which provides joints with an almost frictionless surface during motion(Krishnan et al. 2003; Naka et al. 2005).

Adult articular cartilage is made up of four anisotropic zones: superficial or tangential, middle or transitional, deep or radial and calcified zone (see Figure 2)(Hunziker et al. 2002; Buckwalter et al. 2005). Collagen fibers are arranged in a complex three-dimensional network which differs depending on cartilage zone(Poole et al. 2001; Krishnan et al. 2003; Wu & Herzog 2002).

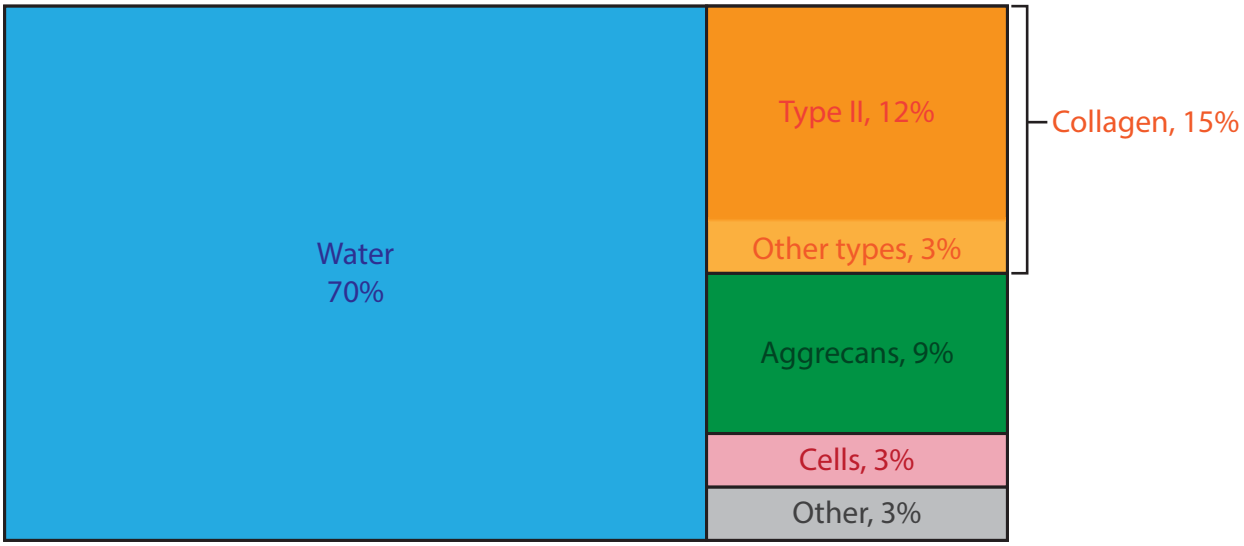

Figure 1. Articular cartilage composition

Water accounts for 70% of articular cartilage. Of the other 30%, collagen, mostly type II, constitutes half of the structural components. While aggrecans represent 9% of the articular cartilages wet weight, chondrocytes only represent 3% (Bronner & Farach-Carson 2007).

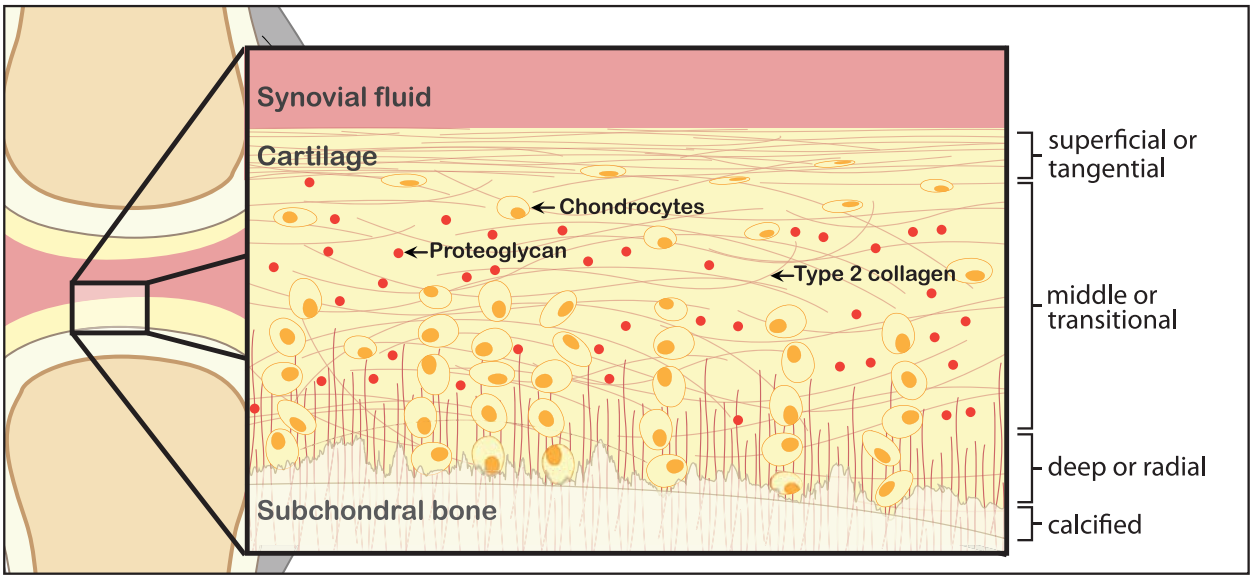

Figure 2. Structural organisation of articular cartilage

Articular cartilage is organized into zones and is mainly composed of water, type 2 collagen, proteoglycans and chondrocytes. Collagen fiber orientation, proteglycan distribution and chondrocyte organization all change according to cartilage zone. Histologically, adult articular cartilage consists of four zones: superficial or tangential, middle or transitional, deep or radial and calcified zone(Buckwalter et al. 2005). The superficial surface provides a highly efficient lubrication mechanism with an extremely low coefficient of friction(Kumar et al. 2001). Deeper layers are largely responsible for the mechanical properties of the articular cartilage, especially its compressive and tensile properties(Kumar et al. 2001). The anisotropic structural organization of the hyaline cartilage seems to be essential for its functional integrity(Hunziker et al. 2002).

Within this network are the proteoglycans, named « aggrecans », which are negatively charged under physiological conditions thus making them hydrophilic and producing rod-like configurations called « proteoglycan aggregates » (Grodzinsky 1983). The mechanical properties of the articular cartilage depend on the interaction between the collagen network, hydrophilic proteoglycan aggregates and water(Quinn et al. 2001). Indeed, experiments have shown that the low hydraulic permeability and high swelling tendency of proteoglycans as well as their confinement within the collagen fiber network are responsible for the joint cartilage's functional integrity(Poole et al. 2001).

1.1.2 Metabolic and biochemical characteristics

Cartilage function and integrity are both maintained by a sole cell type: the chondrocyte. Chondrocytes are highly differentiated cells whose primary purpose is to produce and maintain the ECM in articular cartilage(Muir 1995). Since cartilage is avascular, chondrocytes need to be able to function under very low oxygen conditions because concentration in deep layers can be as low as 1%(Spencer et al. 1990; Henrotin et al. 2005). As such, chondrocytes prefer the anaerobic pathway resulting in lactate production even in aerobic environments(Stockwell 1979; Marcus & Sokoloff 1973). Chondrocytes produce a great variety of molecules to maintain articular cartilage homeostasis. Cytokines, such as Interleukin-1 (IL-1), Interleukin-10 (IL-10), tumour necrosis factor alpha (TNF-α) to name only a few, which have pro- or anti- inflammatory properties through many different signalling pathways are synthesized by cartilage cells(Iannone et al. 2000; Fernandes et al. 2002). These cells are usually long-lived, lasting as long as their owners, and hardly ever divide in adults even though they retain capacity to do so if ECM integrity is degraded(Muir 1995).

The collagen produced by chondrocytes accounts for 50% to 90% of the articular cartilage dry weight(Muir 1995). Collagen is an extracellular protein comprised of three

polypeptide α-chains which each have a tripeptide sequence with glycine in every third position and proline or 4-hydroxyproline frequently in first and/or second position(Budavari 1989). These chains twist tightly into a helix; many molecules combine to form extended rope-like structures named « fibrils »(Muir 1995). In articular cartilage, type 2 collagen (Coll II) represents 80% of the collagen(Bronner & Farach-Carson 2007). Articular collagen has a very long half-life resulting in gradual changes and declined tensile strength over the years(Muir 1995).

Chondrocytes also produce the aggrecans which form the proteoglycan aggregates found embedded in the collagen network. To form these aggregates, many aggrecan molecules will bind to a single chain of hyaluronan, another EMC component synthesized by chondrocytes (see Figure 3)(Muir 1995). The size of the proteoglycan aggregates is key in their immobilisation within the collagen network; bigger sized aggregates more effectively lodge themselves making them immovable(Muir 1995). The affinity of aggrecans for hyaluronan is an important factor in the size of the proteoglycan aggregates and is enhanced when they are processed from a low affinity form to a high affinity form(Sandy et al. 1989). Studies have shown that the processing of aggrecans slows with age(Sandy et al. 1989)

Though articular cartilage has great durability, sometimes providing normal joint function for 80 years or more, it has a limited ability to repair itself and its capacity to do so decreases with age(Buckwalter et al. 2005; Buckwalter & Mankin 1998). This lack of an effective repair response leads to risk of progressive degeneration of the tissue in many individuals older than 40 years (Buckwalter et al. 2000).

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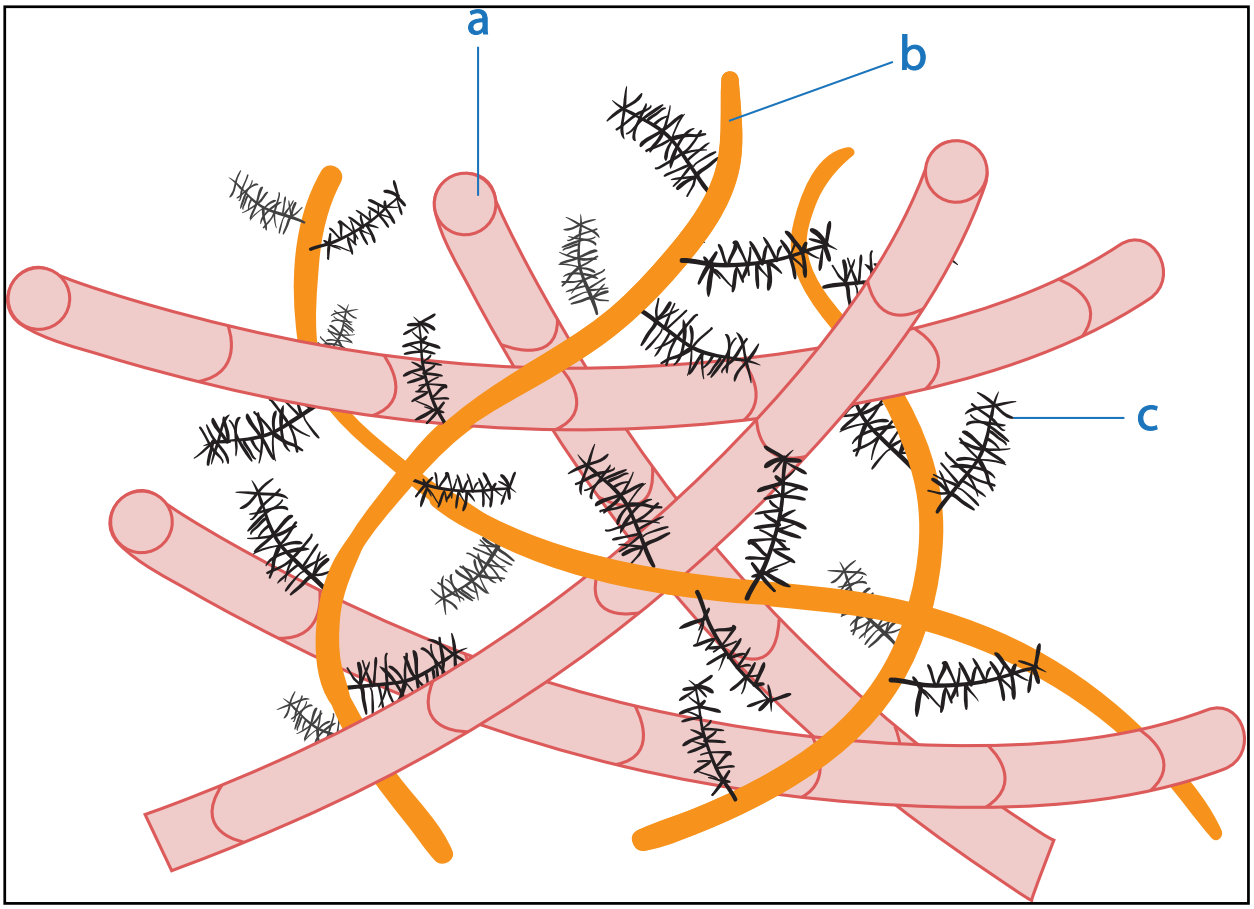


Figure 3. Articular cartilage composition

a. Collagen network consisting of mostly type II collagen. **b.** Chain of hyaluronan to which aggrecans bind to form the proteoglycan aggregates **c.** Aggrecan

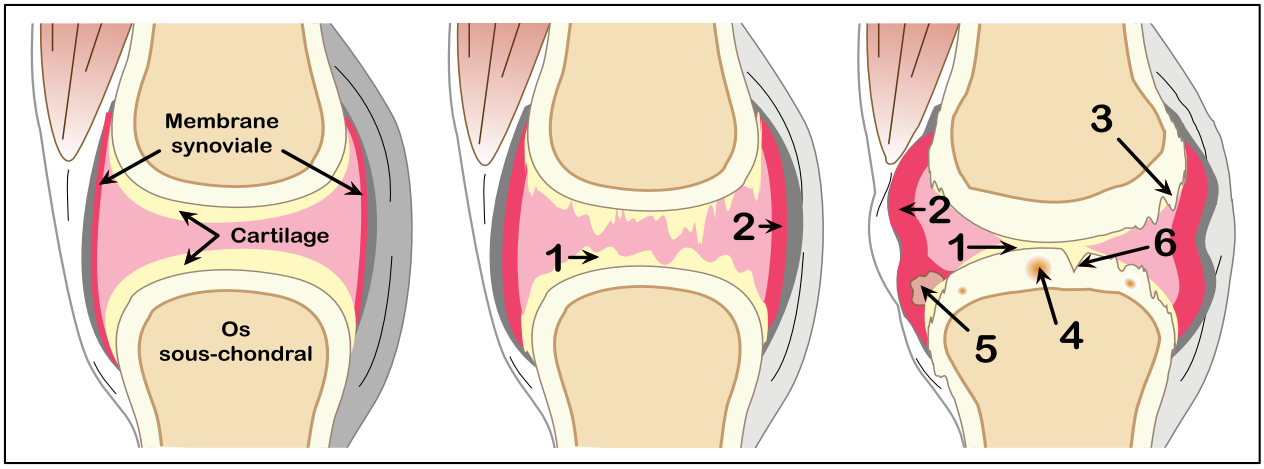
1.2 Osteoarthritis (OA)

1.2.1 Definition and classification

Osteoarthritis (OA), also known as degenerative arthritis or hypertrophic arthritis, is a degenerative joint disorder(Arden & Nevitt 2005).The American College of Rheumatology defines OA as an "heterogeneous group of conditions that lead to joint symptoms and signs which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone at the joint margins."(Bronner & Farach-Carson 2007). It most often affects knee, hip as well as hand joints and is mainly characterized by a progressive and irreversible destruction of cartilage. This causes joint space narrowing, secondary synovial membrane inflammation, osteophytes, subchondral sclerosis, formation of cysts and abnormalities around bone extremities (see Figure 4)(Arden & Nevitt 2005). OA can be categorized into two different types: either primary (or idiopathic), if its origin is not linked to a pathology (usually caused by aging), or secondary, if it can be associated with a specific cause such as a predisposing pathology(Bronner & Farach-Carson 2007).

OA diagnosis is usually assessed using radiography through detection of the above described characteristics(Arden & Nevitt 2005; Spector et al. 1993). For almost fifty years, the Kellgren and Lawrence system has been used by most physicians to determine the severity and stage of the disease(Arden & Nevitt 2005; Spector et al. 1993). This qualitative global grading scale characterizes OA in five grades, from 0, no OA, to 4, severe OA(Arden & Nevitt 2005; Schiphof et al. 2008).

Figure 4. Normal and osteoarthritic knee at early and late stages



In early osteoarthritis, destruction of cartilage (1) and synovial membrane inflammation (2) can be observed. In late or advanced stage, pathology causes osteophyte formation (3), subchondral sclerosis (4), formation of cysts (5) and abnormalities around the bone extremities (6).

There is currently no cure for the disease(Felson, Lawrence, Hochberg, et al. 2000). Indeed, treatment for OA consists of managing and controlling pain to improve both function and quality of life while avoiding therapeutic toxicity(Felson, Lawrence, Hochberg, et al. 2000). The ultimate and definitive treatment when all else has failed or when OA is too severe is the surgical removal of the affected joint(Felson, Lawrence, Hochberg, et al. 2000). However this is used as a last resort since it is highly invasive, costly and has limited durability with patients who keep the prostatic joint for more than 20 years or participate in high-demand activities(Felson, Lawrence, Hochberg, et al. 2000).

Nevertheless, research is being done to find new ways to slow or even reverse OA pathophysiological effects. Indeed, promising research on the use of inflammatory regulatory pathway decoys, such as NF-κB decoys, in rheumatoid arthritis has shown new potential treatment for OA (Miagkov et al. 1998; Makarov 2000). Also being researched is the regenerative capabilities of adipose tissue-derived stromal cells. It has

been shown, both *in vitro* and *in vivo*, that these cells can differentiate into chondrocytes and produce characteristic cartilage matrix molecules which suggest their potential in cartilage tissue regeneration (Awad et al. 2004; Erickson et al. 2002).

1.2.2 Epidemiology of OA

1.2.2.1 Prevalence and incidence of OA

Even though great progress has been made in many fields regarding age- related tissue alteration and diseases, few has been made in articular cartilage changes caused by aging(Martin & Buckwalter 2002). This seems surprising since these changes can increase the risk of synovial joint degeneration with loss of structure and function that can potentially lead to OA. It affects the majority of people by age 65 (60% of men and 70% of women) and 80% of people over 75(Buckwalter et al. 2005; Bronner & Farach-Carson 2007; Arden & Nevitt 2005). According to the Arthritis Foundation, OA affects some 27 million Americans. It is the second most common diagnostic and the number one invalidity cause in elderly people(Peat et al. 2001). In fact, OA is the most common of all joint disorders and accounts for more hospitalizations than rheumatoid arthritis (RA) each year in the United States(Arden & Nevitt 2005). There are few age related diseases that impose a greater burden on the health care system as does OA(Praemer et al. 1992). As mentioned earlier, surgery is the only definitive treatment for OA; this poses a problem because of operation availability. In Canada alone, hip and knee replacement surgeries have more than doubled between 1996 and 2006 with a total of almost 69 000 in 2006(Canadian Institute for Health Information 2008). Waiting times for these surgeries in Canada go from 76 days to 1 year depending on the province and what joint needs replacing(Canadian Institute for Health Information 2006). All of these make OA a major socio-economic problem. Since we are faced with unprecedented rate of population ageing, median age is expected to increase from 26.6 years in 2000 to 37.3 years in 2050, this problem will only get worse unless a solution is found(Arden & Nevitt 2005; United Nations 2002; Steven et al. 2007; Lutz et al. 2008).

1.2.2.2 Risk factors

Risk factors for OA are female gender, exposure to oxidants, overweight/obesity, genetic factors, joint injury, age, increased bone mineral density and intense physical activities(Bronner & Farach-Carson 2007; Arden & Nevitt 2005; Felson et al. 1997; Felson, Lawrence, Dieppe, et al. 2000). Surprisingly, it seems it is not the "wear and tear" that makes aging a risk factor since daily usage appears to have a protective effect on the articular cartilage(Bronner & Farach-Carson 2007). The main problem of aging might rather be linked to a higher rate of cellular senescence, causing problems in EMC replication, repair or maintenance(Bronner & Farach-Carson 2007). As mentioned earlier, the tensile strength of articular collagen changes and decline over the years and processing of aggrecans, giving them higher affinity for hyaluronan, slows with age(Muir 1995; Sandy et al. 1989). Joint injury, overweight/obesity and intense physical activities all add biomechanical stress on the articular cartilage and have been shown in a great number of studies to be important risk factors for knee OA(Blagojevic et al. 2010). Unexpectedly, some studies have shown that smoking might have a small protective effect, though these findings are controversial and are subjects to debate (Blagojevic et al. 2010; Felson et al. 1989).

OA's etiopathogenesis is still relatively unclear; many concepts have been postulated and though biomechanics appear to play a significant role, biochemistry seems as important(Martin & Buckwalter 2002; Dijkgraaf et al. 1995).

1.3 Pathophysiology of OA

Regardless of what causes it initially, OA starts with an imbalance in the synthesis and degradation of the ECM by chondrocytes (Dijkgraaf et al. 1995). In healthy articular cartilage, chondrocytes are responsible for maintaining ECM homeostasis(Martin & Buckwalter 2002). The anabolic functions of chondrocytes are usually controlled by cytokines and growth factors(Sandell & Aigner 2001). These factors include transforming growth factor beta (TGF-β), insulin-like growth factor I (IGF-I) and bone morphogenetic proteins (BMPs)(Sandell & Aigner 2001). In the beginning of OA, osteoarthritic cartilage shows increased water content, early loss of proteoglycans as well as alterations in both size and arrangement of collagen fibers(Dijkgraaf et al. 1995; Goldring 2000). Repair attempts lead to accelerated cartilage turnover which means increased cell proliferation and matrix synthesis(Dijkgraaf et al. 1995).

The chondrocytes repair response is also thought to be increased: (1) by greater diffusion on growth factors cartilage due to its loss of integrity, (2) by release of previously ECM component-bound growth factors and (3) by chondrocyte upregulation of growth factor expression, such as IGF-1(Dijkgraaf et al. 1995). Unfortunately, as the pathology evolves, the increased synthesis cannot sufficiently compensate for the increased degradation resulting in tissue degeneration (see Figure 5)(Dijkgraaf et al. 1995; Sandell & Aigner 2001).

In early OA, the cytokine/growth factor balance responsible for normal homeostasis is disrupted and an increase in both inflammatory and catabolic cytokines can be measured in OA synovial fluids(Sandell & Aigner 2001; Goldring 2000). Proinflammatory cytokines, such as IL-1, TNF-α, IL-17 and IL-18, are known to cause an important increase in proteinase activity. In OA, they are produced by two types of cells: activated synovial cells, leading to proinflammatory cytokine presence in synovial fluid thus acting on cartilage resident cells in a paracrine manner, as well as chondrocytes, which leads to autocrine activation(Fernandes et al. 2002; Sandell & Aigner 2001; Goldring 2000). They are responsible for greater matrix metalloproteinases (MMPs) synthesis, an increase in aggrecanases, a decrease in MMP enzyme inhibitors and in ECM production(Mort & Billington 2001; Henrotin et al. 2002; Dean 1991). Proinflammatory cytokines also induce cyclooxygenase-2 (COX2) and inducible nitric oxide synthase (iNOS) through nuclear factor kappa B (NFκB) activation which lead to an increased release of both prostaglandin E2 (PGE_2) and nitric oxide (NO)(Roman-Blas & Jimenez 2006; Amin et al. 1997). TNF-α also stimulates synthesis of phospholipase A_2 (PLA_2) by chondrocytes.

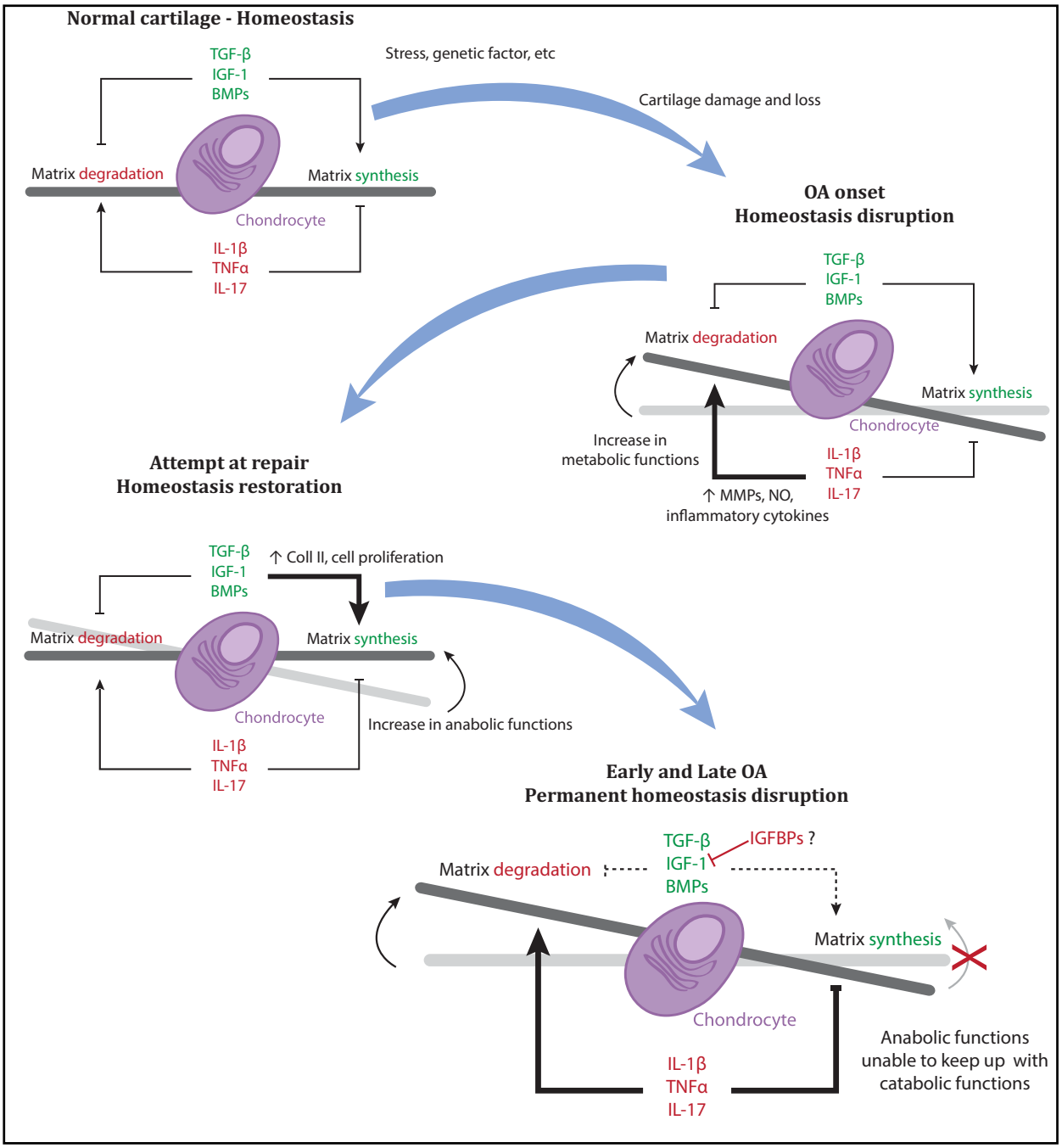


Figure 5. Osteoarthritis and matrix homeostasis

In normal cartilage, chondrocytes maintain matrix homeostasis. But when joint suffers cartilage damage and/or loss due to one of many factors (mechanical stress, genetic factors, etc.), the fragile equilibrium between matrix synthesis and degradation is disrupted. This onset of OA leads to upregulation of matrix metalloproteinases (MMPs) and inflammatory cytokines (interleukine 1β (IL-1β), IL-17 and tumour necrosis factor-α (TNF-α)) which increases nitrous oxide (NO) generation(Sandell & Aigner 2001; Goldring 2000). This is followed by attempts to repair cartilage by increasing anabolic functions, thanks to growth factors (transforming growth factor beta (TGF-β), insulin-like growth factor I (IGF-I) and bone morphogenetic proteins (BMPs)) which results in type II collagen (Coll II) synthesis and reorganization as well as in chondrocyte proliferation(Dijkgraaf et al. 1995). Unfortunately, as OA progresses, anabolic functions are unable to keep up with tissue degradation.

1.3.1 Roles of cytokines in OA

Proinflammatory cytokines are cell-signaling molecules that are responsible for inducing inflammation mediators such as iNOS, COX-2, PLA_2, etc(Dinarello 2000; Feldmann & Saklatvala 2001). It is a widespread belief that proinflammatory cytokines play a crucial role in the genesis and development of OA(Poole et al. 2001; Kobayashi et al. 2005; Hedbom & Häuselmann 2002). There are many proinflammatory cytokines, including many ILs, TNFs, interferons and colony-stimulating factors, but among these, two appear to be most directly implicated in the pathological processes of OA, IL-1β and TNF-α(Dinarello 2000; Kobayashi et al. 2005). Through activation of many transcription factors, such as NF-κB and the activator protein (AP)-1, these two act synergistically and can induce synovial cells and chondrocytes to synthesize other proinflammatory cytokines including IL-8 and IL-6(Fernandes et al. 2002).

1.3.1.1 IL-1β

The IL-1 gene family consists of three genes: IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra)(Dinarello 1996). The two former are agonists while the latter is a specific receptor antagonist(Dinarello 1996). IL-1α represents only about 10% of IL-1 agonist proteins produced by LPS-activated human monocytes and its processing and release remain to be understood(Feldmann & Saklatvala 2001). IL-1β accounts for about 90%(Feldmann & Saklatvala 2001). Both exist in two forms: mature (17,5 kDa) and precursor (pro-IL-1α or pro-IL-1β) (31 kDa)(Fernandes et al. 2002; Goldring 2000). In the case of IL-1β, the mature (or active) form is generated through pro-IL-1β cleavage by IL-1β converting enzyme (ICE also known as caspase-1) and can be found in synovial membrane, synovial fluid and cartilage(Fernandes et al. 2002; Feldmann & Saklatvala 2001).

Even though there are two types of cell surface receptors (IL-1R), namely type I (IL-1RI) and type II (IL-1RII), it appears IL-1β induces inflammation solely as a result of its association to the first(Feldmann & Saklatvala 2001). Indeed, signal transduction of IL-

1 can only be achieved through IL-1α or IL-1β binding to IL-1RI(Dinarello 1996) (see Figure 6a). Type II IL-1R (IL-1RII) has been described as being a "decoy" receptor for IL-1β since it binds IL-1β tightly but, due to a short signal transducing cytosolic domain, cannot lead to cell signaling(O'Neill & Dinarello 2000; Colotta et al. 1994) (see Figure 6b). Both IL-1RI and IL-1RII can be found in soluble form (IL-1sRI and IL-1sRII) in body fluids(Dinarello 1996). While IL-1RI has greater affinity with IL-1α than with IL-1β, IL-1RII binds IL-1β with a lot more affinity than IL-1α(Dinarello 1996). IL-1RII, in both cell-bound and soluble form, seems to be an efficient IL-1β trap due to high affinity and almost irreversible binding caused by a very slow dissociation rate (2 hours)(Dinarello 1996).

An IL-1R accessory protein (IL-1R-AcP) has also been identified and seems to be vital to IL-1β signaling(Feldmann & Saklatvala 2001). Indeed, IL-1β signal transduction necessitates the formation of a IL-1RI/ IL-1R-AcP/ IL-1β complex which probably leads to dimerization of the IL-1RI and IL-1R-AcP cytosolic domains (see Figure 6a)(Feldmann & Saklatvala 2001; O'Neill & Dinarello 2000; O'Neill et al. 2003). IL-1β appears to first bind IL-1RI with low affinity forming a IL-1β/IL-1RI complex which allows IL-1R-AcP to dock to the newly formed complex whose binding affinity is now high(Dinarello 1996). IL-1 activation results in nuclear translocation of NF-κB and AP-1(Dinarello 2000). The biochemical events induced by IL-1 take place within a few minutes of its activation; among these is phosphorylation of PLA_2 AP which leads to a rapid release of arachidonic acid (AA) (Dinarello 2000).

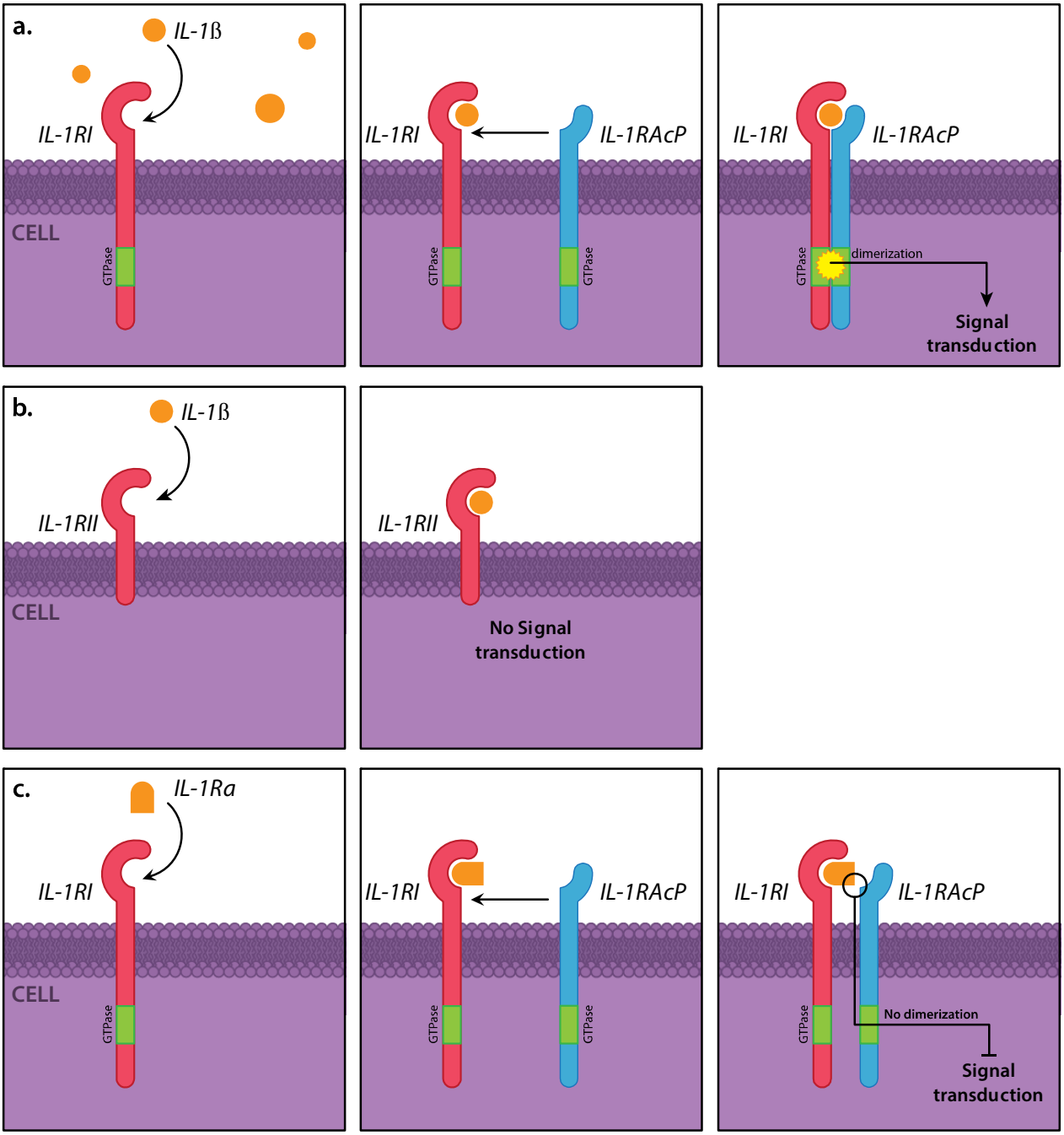


Figure 6. Interleukine-1beta (IL-1β) signal transduction

a. Interleukine-1beta (IL-1β) binds its receptor, IL receptor type I (IL-1RI) with low affinity. IL-1R accessory protein (IL-1R-AcP) then associates with the IL-1β/ IL-1RI complex with a high affinity. Formation of the IL-1RI/ IL-1R-AcP/ IL-1β complex leads to signal transduction through dimerization of cytosolic domains.
b. IL-1β binds its receptor, IL receptor type II (IL-1RII) with high affinity. However, due to a short signal transducing cytosolic domain no signal can be transduced.
c. IL-1R antagonist (IL-1Ra) avidly binds to IL-1RI. IL-1R-AcP cannot associates with the IL-1Ra/ IL-1RI complex, thus leading to no signal transduction.

Another related protein is the IL-1Ra which is a natural inhibitor of IL-1(Kobayashi et al. 2005). It acts by binding to IL-1R without inducing signal transduction since IL-1R-AcP cannot bind the IL-1Ra/ IL-1RI complex(Feldmann & Saklatvala 2001) (see Figure 6c). IL-1Ra is a soluble 22 kDa glycosylated protein which can also be found in a non-glycosylated 17kDa form(Kobayashi et al. 2005). It is under this last form that IL-1Ra binds IL-1R(Dinarello 1996). IL-1Ra has 26% homology with IL-1β (which is greater than the homology between IL-1β and IL-1α) (Dinarello 1996). However, contrary to IL-1β, IL-1Ra has much more affinity with IL-1RI than with IL-1R2(Dinarello 1996). IL-1RI occupancy by IL-1Ra very effectively prevents IL-1 signal transduction(Dinarello 1996).

1.3.1.2 TNF-α

The TNF superfamily comprises many trimeric cytokines and cell surface proteins (at least 19) such as Fas Ligand (FasL), lymphotoxin-α (LTα), receptor-activator of NF-κB ligand (RANKL), CD40 ligand (CD40L), TNF-α, etc.(Baud & Karin 2001; Popa et al. 2007). In mammalian cells, the TNF superfamily plays an essential role in a broad array of biological processes: inflammation, cell activation, apoptosis, host defense, autoimmunity, etc (Keystone & Ware 2010). TNF-α can bind two structurally distinct receptors, type I (TNF-RI) and type II (TNF-RII)(Tracey et al. 2008). The pleiotropic cytokine TNF-α has a complex role in inflammation which is very similar to IL-1 since they share many pathways (Popa et al. 2007). In fact, both cytokines will often synergize for NO induction, resulting in cell death (Dinarello 2000).

TNFR1 activation leads to two distinct signaling pathways: the primary one activates both NF-κB and AP-1 through inhibitors of κB kinase (IKK) and Jun NH_2-terminal Kinase (JNK) while the second induces apoptosis in a caspase-8 and caspase-3 manner(Baud & Karin 2001; Tracey et al. 2008). As for TNFR2, its activation always results NF-κB and AP-1 signaling pathways(Baud & Karin 2001). Activation of NF-κB suppresses the apoptosis pathway, as long as it is not compromised; thus TNF-α signaling will usually lead to a proinflammatory response (see Figure 8)(Tracey et al. 2008). The extracellular domain fragments of each receptor can be shed and they are then said to be in soluble form: soluble TNFR-1 and -2 (sTNRF-1 and -2)(Sharma & Anker 2002). Though there function is yet unknown, at high concentrations, sTNFRs inhibit TNF activity as they trap TNF-α(Sharma & Anker 2002).

1.3.1.3 Other proinflammatory cytokines

Many other proinflammatory cytokines have been described and are thought to play a role in OA, namely IL-8, IL-17 and IL-18(Fernandes et al. 2002). All three enhance matrix degradation and ROS generation through either upregulation of IL-1β and/or TNF-α or suppression of aggrecan synthesis(Fernandes et al. 2002; Bronner & Farach-Carson 2007). IL-6 also seems to play a role in OA as it causes an increase in IL-1Ra, sTNFR, and tissue inhibitor of metalloproteins (TIMP) as well as enhances immune cell function and inflammation(Bronner & Farach-Carson 2007) [. On the other hand, when IL-6 interacts with its soluble receptor (sIL-6R), it leads to upregulation of MMPs and A disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) as well as downregulation of Coll II gene (COL2A1) and aggrecan(Bronner & Farach-Carson 2007).

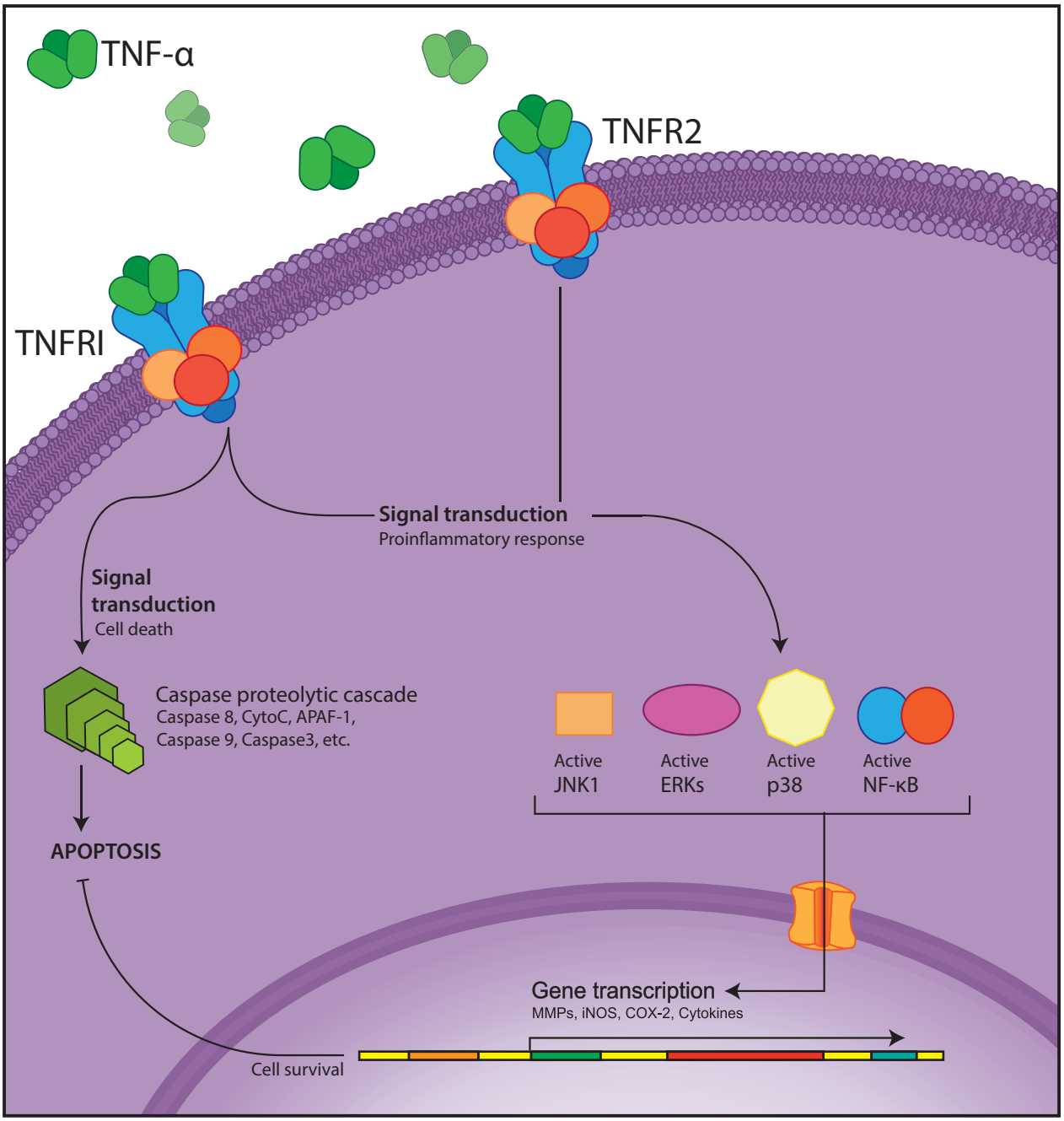


Figure 7. Tumor Necrosis Factor-α (TNF-α) signal transduction

Tumor Necrosis Factor-α (TNF-α) can bind either TNF Receptor 1 (TNFRI) or 2 (TNFRII). TNFRI activation induces to the Caspase proteolytic cascade through activation of Caspase 8 which leads in Cytochrome-C (CytoC) release and activation of Apoptotic Protease Activating Factor-1 (APAF-1). In turn, APAF-1 will recruit Caspase 9 and activate Caspase 3 which ultimately results in apoptosis. TNFRI activation also activates Jun NH2-terminal Kinase -1 (JUNK1), Mitogen-Activated Protein Kinases (MAPK) p38 and Extracellular signal-Regulated Kinases (ERK) and Nuclear Factor –KappaB (NF-κB); all of these result in a proinflammatory response (Activation of Metalloproteinases (MMPs), induction on inducible Nitrous Oxide (iNOS), cyclooxygenase-2 (COX-2) and many protinflammatory cytokines). The proinflammatory response suppresses cell death. TNFRII activation always leads to the proinflammatory response(Baud & Karin 2001; Popa et al. 2007; Keystone & Ware 2010; Tracey et al. 2008; Sharma & Anker 2002).

1.3.1.4 NF-κB pathway

The NF-κB family of transcription factors play a central role in the signaling pathways of stress-induced apoptosis, inflammation and immune signaling through innate and adaptive immune responses(Roman-Blas & Jimenez 2006). This family has five members that associate to form a variety of homo- and hetero- dimers: RelA (p65), ReloB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100)(Roman-Blas & Jimenez 2006; Tak & Firestein 2001). The RelA (p65) and NF-κB1 (p50/p105) heterodimer is the most widespread activated form of NF-κB(Tak & Firestein 2001).

In OA, it mediates key events in the inflammatory response by chondrocytes. These events eventually lead to the progressive ECM destruction characteristic of OA(Agarwal et al. 2004). NF-κB is known to regulate more than 150 genes; these include genes encoding cytokines, for example TNF-α, IL-1β and IL-6, inducible enzymes, such as COX-2 and iNOS, and both pro- and anti- apoptotic molecules(Roman-Blas & Jimenez 2006). It is present in the cytoplasm in an inactive form(Roman-Blas & Jimenez 2006; Tak & Firestein 2001). In this state, the NF-κB dimer is associated with regulatory proteins known as inhibitors of κB (IκB): IκBα, IκBβ, IκBε and IκBγ(Roman-Blas & Jimenez 2006). NF-κB can be activated by many stimuli, but in OA, the most significant ones are cytokines, including TNF-α and IL-1β, and free radicals(Roman-Blas & Jimenez 2006). These trigger signalling pathways that ultimately lead IκB phosphorylation by IKK(Roman-Blas & Jimenez 2006; Tak & Firestein 2001). Though the exact mechanism through which the IKK complex is activated by cytokines remains unknown, when activated, IKK initiates IκBα phosphorylation at specific serine residues(Roman-Blas & Jimenez 2006; Tak & Firestein 2001). NF-κBs nuclear localization signal is the revealed and the dimers are then free to translocate into the nucleus, to bind to κB enhancer elements of target genes and stimulate gene transcription (see Figure 8) (Roman-Blas & Jimenez 2006; Tak & Firestein 2001).

NF-κB is implicated in many inflammatory diseases such as OA, multiple sclerosis, asthma, type 2 diabetes, etc(Roman-Blas & Jimenez 2006). In OA patients, IKK is constitutively expressed and its activation by TNF-α and IL-1β is crucial for NF-κB mediated cytokine, collagenase, iNOS and COX-2 and induction(Roman-Blas & Jimenez 2006; Tak & Firestein 2001).

Thought NF-κB activation through IL-1β and TNF-α activation is well known, it has been shown that biomechanical signals can be converted into biochemical signals by cells and therefore also activate NF-κB(Agarwal et al. 2004). In experiments conducted with rabbit articular cartilage grown on flexible membranes, it has been shown that high magnitude cyclic tensile strain (15-18% equibiaxial strain) have the same effect on NF-κB activation than IL-1β since it induced rapid NF-κB p50-p65 heterodimer nuclear translocation, thus upregulating proinflammatory gene expression, matrix degradation and decreasing matrix synthesis(Agarwal et al. 2004). NF-κB is also known to be redox-sensitive(Henrotin et al. 2003).

Taking into account that

OA is an age-related disease; chondrocyte senescence leads to mitochondrial degeneration, therefore to an increase in free radical production, such as superoxide anion ($O_2^{\bullet}$) thus adding redox stress to the articular joint. On the other hand, mechanical strain in known to be an important risk factor for OA. This indicates that both redox and biomechanical signal activation pathways may greatly contribute in OA pathogenesis(Martin & Buckwalter 2002).

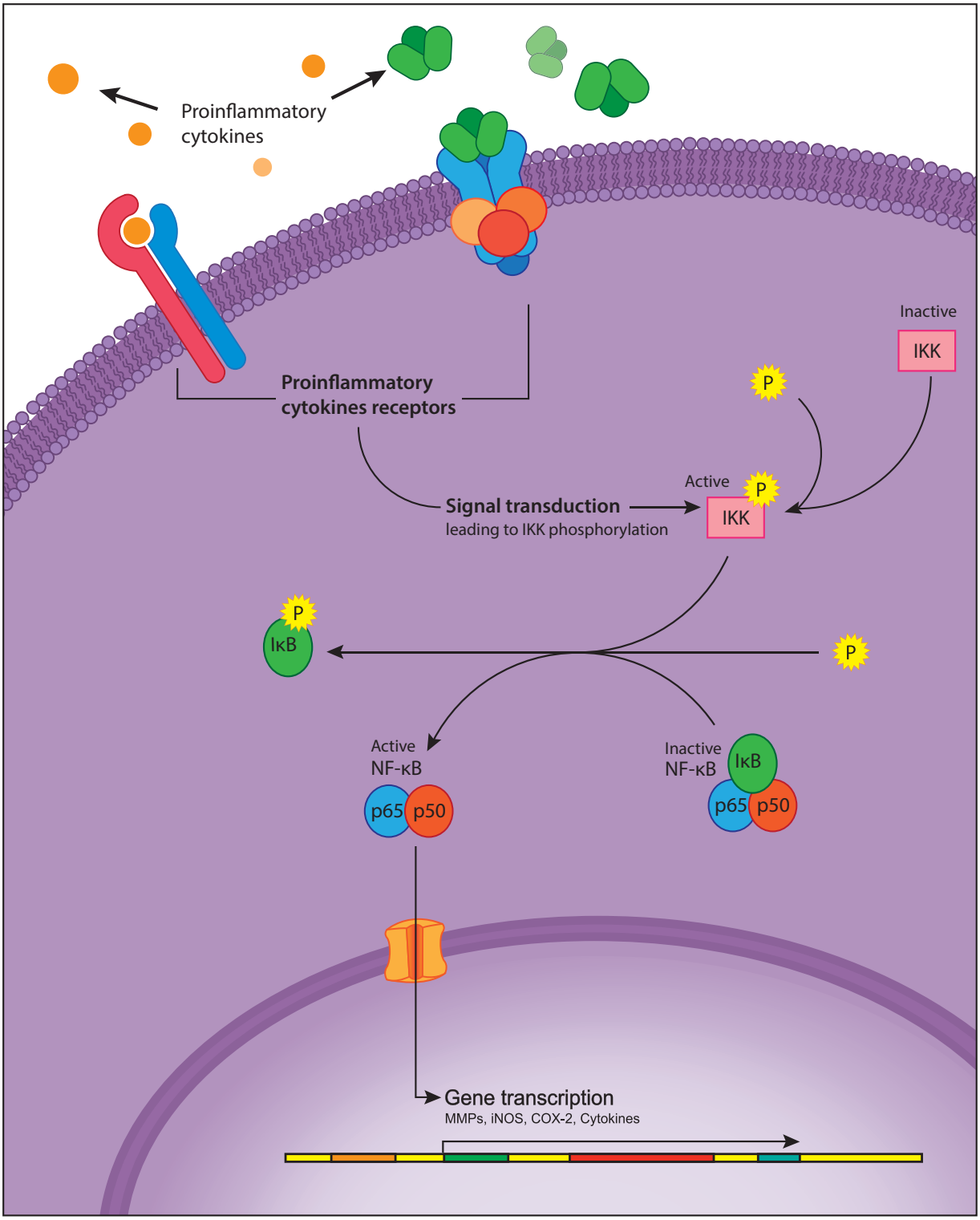


Figure 8. Nuclear factor-kappaB (NF-κB) cytokine activated pathway

Proinflammatory cytokines bind to their receptors which lead to an unknown signal transduction that result in inhibitors of κB kinase (IKK) activation through its phosphorylation. IKK activation initiates inhibitors of κB (IκB) phosphorylation which releases NF-κB dimer. NF-κB then translocates into the nucleus to stimulate gene transcription (Activation of Metalloproteinases (MMPs), induction on inducible Nitric Oxide (iNOS), cyclooxygenase-2 (COX-2) and many protinflammatory cytokines)(Dean 1991; Sharma & Anker 2002).

1.3.1.5 COX-2 and PGE_2

Cyclooxygenase (COX) is the enzyme responsible for prostaglandin (PG) synthesis. Also called prostaglandin H_2 synthase (PGHS), COX converts AA into prostaglandins G2 (PGG_2), then PGH_2 that can subsequently be converted to a variety of eicosanoids, more specifically prostanoids, which include PGE_2, PGF_{2a}, PGI_2 and thromboxane (TX) A2 (see Figure 9)(Vane et al. 1998; Cryer & Feldman 1998). Though it was long thought that COX existed in only one form, two COX isoforms exist: COX-1 and COX-2(Dubois et al. 1998). Both have the same enzymatic activity and are very similar in structure(Vane et al. 1998). What differentiates the two is that the first is constitutive and present in almost all type of cells while the latter is inducible and normally absent from cells(Dubois et al. 1998). Therefore COX-1 is responsible of producing homeostatic levels of PGs allowing regulation of physiological effects such as platelet aggregation (blood clot formation)(Dubois et al. 1998). Synthesis of COX-2 can be stimulated by a great number of growth factors and proinflammatory cytokines, such as IL-1β and TNF-α, through NF-κB inhibition(Amin et al. 1997; Gupta & DuBois 2001). In fact, PGs most important action is their role in inflammation; more importantly, PGE_2 release is a key event in the swelling/redness, pain and fever caused by inflammation(Clària 2003). COX-2 has been shown to be responsible for PG synthesis in inflamed joint tissues and its induction has been observed in OA cartilage and synovial tissue(Clària 2003). These observations that have led searchers to investigate COX-2 selective inhibitors as an anti-inflammatory treatment in OA (Clària 2003).

Figure 9. Diagram of cyclooxygenase (COX) role in eicosanoid metabolism

Formation of prostaglandin (PG) from arachidonic acid. Cyclooxygenase (COX) -1 and -2 oxygenate arachidonic acid to form PGG_2 which will be reduced to PGH_2. PGH_2 will then be transformed into various prostaglandins by their respective synthase(Vane et al. 1998).

1.3.2 Matrix metalloproteinases (MMPs) and aggrecanases

MMPs are a family of degradative enzymes which target ECM components and are believed to have a central role in tissue remodelling and repair but, when in excessive expression or in lack of inhibition, they are thought to greatly contribute to OA pathogenesis(Sandell & Aigner 2001; Shapiro 1998). They are produced in an pro-enzyme form and are activated by a proteolytic cleavage by cell surface associated plasmin or by other MMPs(Knäuper et al. 1996; MURPHY et al. 1999). There are many MMPs, such as 1,2,3,7,8,13 and 14, only to name a few, and they consist of different collagenases, stromelysins, gelatinases and matrilysin(Shapiro 1998; Shlopov et al. 1997).

In OA, levels of MMP-3 (a stromelysin) MMP-1, MMP-8 and MMP-13 (all three collagenases) are elevated (Sandell & Aigner 2001; Goldring 2000; Shlopov et al. 1997). In fact, though all three collagenases seem to play a key role in OA, Coll II degradation seems to be mainly caused by MMP-13(Shlopov et al. 1997; Billinghurst et al. 1997). IL-1β stimulation can enhance MMP-13 transcription because of the parallel activation of kinase cascades regulating NF-κB and AP-1(Tak & Firestein 2001). MMP-3 is responsible for degrading many ECM molecules, including proteoglycan and Coll II(Takahashi et al. 1999).

Some enzymes, named « aggrecanases », are responsible of aggrecan proteolytic degradation; it is the case of some members of the ADAMTS family namely ADAMTS-4 (also known as aggrecanase-1) and ADAMTS-5 (aggrecanase-2). Although both ADAMTS-4 and ADAMTS-5 can degrade aggrecans and affect articular cartilage integrity, studies with ADAMTS-4 and/or -5 knock-out mice seem to show that only the latter is associated with increased susceptibility to OA(Tang 2001; Majumdar et al. 2007; Glasson et al. 2004; Glasson et al. 2005).

The activation of MMPs can be inhibited by TIMPs. TIMPs do so by forming a high affinity complex(Pelletier et al. 1990). It seems that conditions in OA tissues favor MMP synthesis and activation over inhibition. Indeed, a lack of sufficient TIMP to counter increased MMP production might be a contributing factor in OA articular cartilage degradation(Pelletier et al. 1990).

1.3.3 Reactive oxygen species (ROS)

Cytokines as well as mechanical stress can both stimulate reactive oxygen species (ROS) production by chondrocytes(Henrotin et al. 2005). ROS interact strongly and quickly with many molecules, both organic and inorganic, irreversibly changing or even destroying them(Bedard & Krause 2007). These include various proteins, lipids and nucleic acid(Bedard & Krause 2007). O_2^- and NO are the two main ROS generated by articular cartilage cells(Henrotin et al. 2005). Members of the nicotinamide adenine dinucleotide phosphate (NADPH) Oxidase (NOX) family reduce oxygen (O_2) to O_2^- making them big ROS producers(Bedard & Krause 2007). Both IL-1β and NO can activate NOX(Kaur et al. 2004). O_2^- and NO play an important role in pathophysiology since derivative radicals such as hydrogen peroxide (H_2O_2) and proxynitrite ($ONOO^-$) as well as hydroxyl radical (OH^-) are created when in presence of iron(Henrotin et al. 2003; Henrotin et al. 2005). In OA, production of ROS is such that antioxidants capacity to detoxify them is insufficient, causing oxidative stress to articular cartilage, thus damaging it. ROS have been shown to be involved in cartilage degradation through intracellular signaling regulation and through direct attack of ECM molecules, proteoglycan and collagen molecules in particular(Henrotin et al. 2003). Various transcription factors, including NF-κB, are redox-sensitive through modification of conserved cysteines(Henrotin et al. 2003). ROS can also modify protein degradation by inhibiting the ubiquitin pathway(Henrotin et al. 2003). One family of ROS which has been shown to be very implicated in OA is the reactive nitrogen species (RNS) family.

1.3.4 Nitric oxide (NO) and inducible NO synthase (iNOS)

RNS formation in chondrocytes starts with NO which will then lead to $ONOO^-$ (Henrotin et al. 2005)[17]. NO is synthesized by its enzyme, NO synthase (NOS), which has three isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducile NOS (iNOS) (Henrotin et al. 2005). Only the two latter are present in chondrocytes. While eNOS is expressed constitutively, iNOS is, as its name suggests, an inducible form. As for ROS stimulation, iNOS expression can be induced by either mechanical stress or cytokines(Henrotin et al. 2005). Since OA is characterized by an overproduction of cytokines and an increase in mechanical stress at the articular cartilage level, it is not surprising that many studies has shown increased levels of iNOS in OA cartilage when compared to normal cartilage(Pelletier et al. 2001). Indeed, OA cartilage produce large amounts of NO(Henrotin et al. 2005; Henrotin et al. 2003; Pelletier et al. 2001; Pelletier et al. 1998). NO combines with O_2^- to form $ONOO^-$(Henrotin et al. 2005). NO and $ONOO^-$ play an important role in OA. They affect cartilage matrix synthesis by being responsible for inhibition of aggrecan and COLL II synthesis and they increase the IL-1β inhibitory effect on EMC component synthesis(Henrotin et al. 2003). They also contribute to cartilage matrix breakdown by inducing chondrocyte apoptosis, increasing MMP-3 and MMP-13 mRNA levels in chondrocytes while inhibiting TIMPs(Henrotin et al. 2003). Since they upregulate proinflammatory cytokines, their production causes an amplification of cartilage catabolism, and all subsequent problems such as inflammation, swelling and pain, through a feedback loop (see Figure 10)(Pelletier et al. 2001).

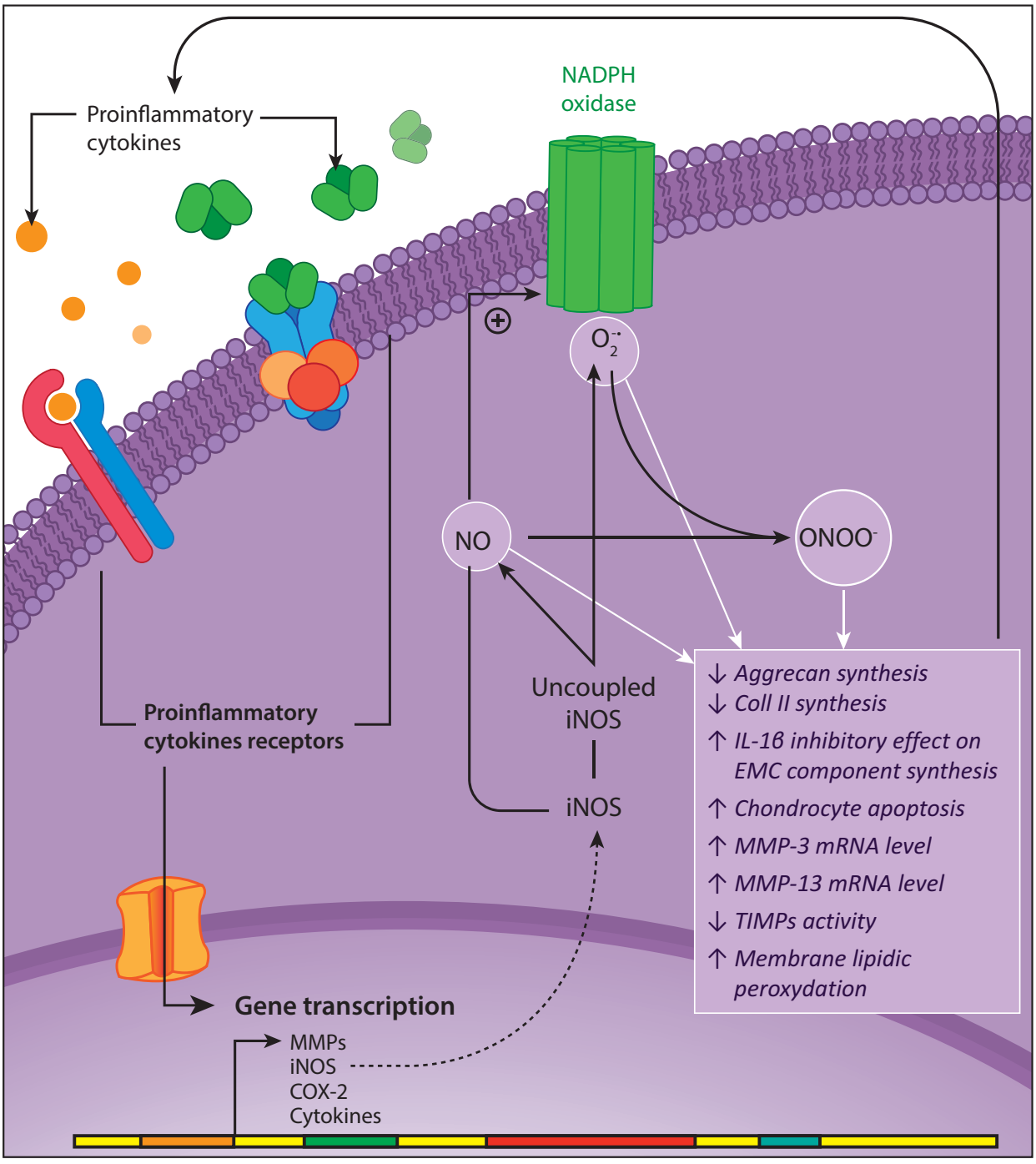


Figure 10. Role of inducible nitric oxide synthase (iNOS) in OA

Proinflammatory cytokines lead to inducible nitric oxide synthase (iNOS) expression. iNOS stimulates reactive oxygen species (ROS) production such as nitric oxide (NO), superoxide anion (O_2^-) and proxynitrite ($ONOO^-$)(Henrotin et al. 2003). NO, O_2^- and $ONOO^-$ contribute to cartilage matrix degradation by inhibiting aggrecan and type II collagen (COLL II) synthesis, increasing the interleukine-1β (IL-1β) inhibitory effect on matrix component synthesis, inducing chondrocyte apoptosis, increasing metalloproteinase (MMP)-3 and MMP-13 mRNA levels in chondrocytes while inhibiting tissue inhibitor of metalloprotease (TIMPs) (Henrotin et al. 2003). They also upregulate proinflammatory cytokines, which causes an amplification of cartilage catabolism, and stimulate lipidic peroxidation of cell membrane(Pelletier et al. 2001; Halliwell & Chirico 1993).

The iNOS enzyme itself might also contribute to ROS formation. Indeed, limitation of substrate availability leads to uncoupling of NOS and ultimately to ROS formation (Gielis et al. 2011). When NOS is in its uncoupled form, electron transfer can result reduction of molecular oxygen, and NO which can react together to form $ONOO^-$ (Griffith & Stuehr 1995). $ONOO^-$ is also known for lipid peroxidation (LPO) of the cell membrane, composed of polyunsaturated fatty acids (PUFAs)(Radi et al. 1991). LPO produces lipid peroxides through a free radical chain reaction which will in turn be decomposed to produce epoxides, ketones, acids and aldehydes (see Figure 11 and Figure 12) (Halliwell & Chirico 1993).

1.4 4-Hydroxynonenal (HNE)

1.4.1 Synthesis and characteristics of HNE

One of the many effects of oxidative stress caused by the ROS and RNS family is LPO of the polyunsaturated fatty acids contained in the cell's membrane (Radi et al. 1991). The cell membrane is a lipid bilayer whose main function is to control the metabolite and electrolyte concentration gradient between the cell and it's surrounding through modulation of the membrane's permeability. Lipid damage can thus affect these gradients and affect cell function(Hogg & Kalyanaraman 1999). Of course, cells have the ability to repair or replace damaged lipids, but since LPO is a rapid chain reaction, normal lipid turnover can be insufficient(Hogg & Kalyanaraman 1999). Moreover, products of LPO, such as hydroperoxides (LOOH), alcohols and aldehydes can affect both cellular functions and protein expression [88]. PUFAs, such as arachidonic acid, are more sensitive to oxidation because their methylene hydrogens are more susceptible to hydrogen abstraction by ROS and RNS than those from saturated lipids(Hogg & Kalyanaraman 1999).

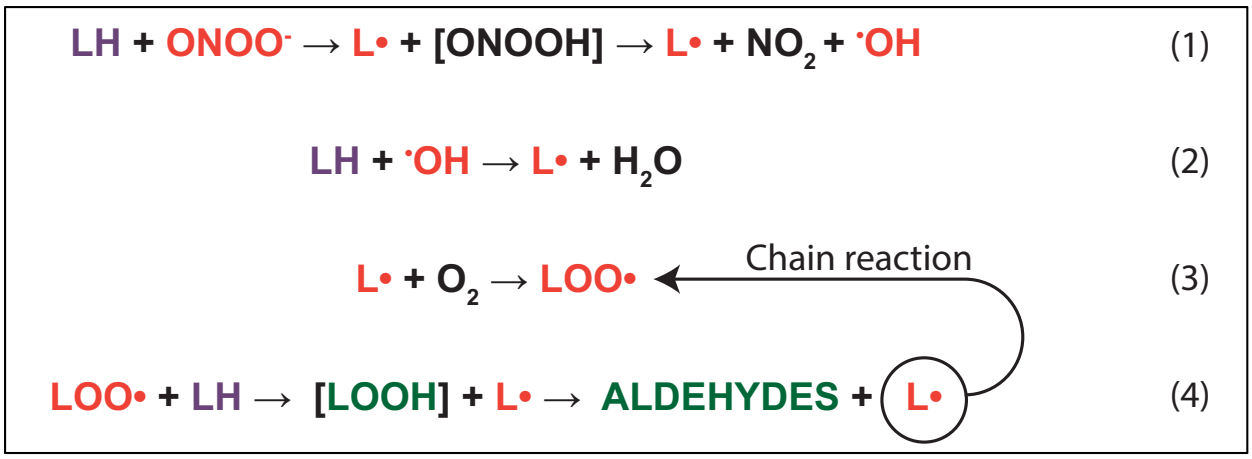


Figure 11. Molecular equations of lipidic peroxidation

Overview of aldehyde generation through lipidic peroxidation (Hogg & Kalyanaraman 1999; Herdegen & Delgado-Garcia 2004).

Equations 1 and 2 show how $ONOO^-$ and $^{\bullet}OH$ remove hydrogen from unsaturated lipids (LH) to form a lipid radical (L•)(Hogg & Kalyanaraman 1999; Herdegen & Delgado-Garcia 2004). The lipid radical produced subsequently reacts with oxygen to generate a lipid peroxyl radical (LOO•), which is key to the propagation of LPO through the cell membrane (eq.3) (Hogg & Kalyanaraman 1999). The lipid peroxyl radical produced will then remove a hydrogen to the neighboring unsaturated lipid to regenerate a lipid radical, which will lead to the LPO chain reaction, and LOOH which, due to its unstableness, will quickly fragment into reactive aldehydes (eq. 4) (see Figure 11 and Figure 12)(Hogg & Kalyanaraman 1999). Aldehydes produced by LPO are believed to be the main culprits in cell damages caused by oxidative stress(Esterbauer et al. 1991). Because aldehydes are more stable than free radicals, they can diffuse in and out of cells and target cells and structures far from their point of origin(Dalle-Donne et al. 2006). In these cases, aldehydes are secondary messengers to primary reactions(Dalle-Donne et al. 2006). The most important aldehydes generated by LPO are

malondialdehyde (MDA) and 4-Hydroxynonenal (HNE), the former being the most abundant lipid aldehyde while the latter is the one who which is most responsible for the cytopathologic effects of oxidative stress(Uchida 1999). As many other aldehydes, these two can react with various biomolecules such as phospholipids, proteins and even ribonucleic and deoxyribonucleic acids (RNA and DNA)(Dalle-Donne et al. 2006).

HNE is an amphiphilic compound since it has both hydrophilic and lipophilic properties, though it has greater affinity for lipids than for water(Poli et al. 2008). It is known as a highly reactive molecule due to its three functional groups: a hydroxyl group (OH), a carbon-carbon (CC) double bond as well as a carbonyl group (CO) (see Figure 13)(Poli et al. 2008). Together, these three groups frequently act synergistically so that HNE can react to a great number of biomolecules, especially those with amino residues (cysteine, lysine, histidine and rarely arginine) or thiol groups.

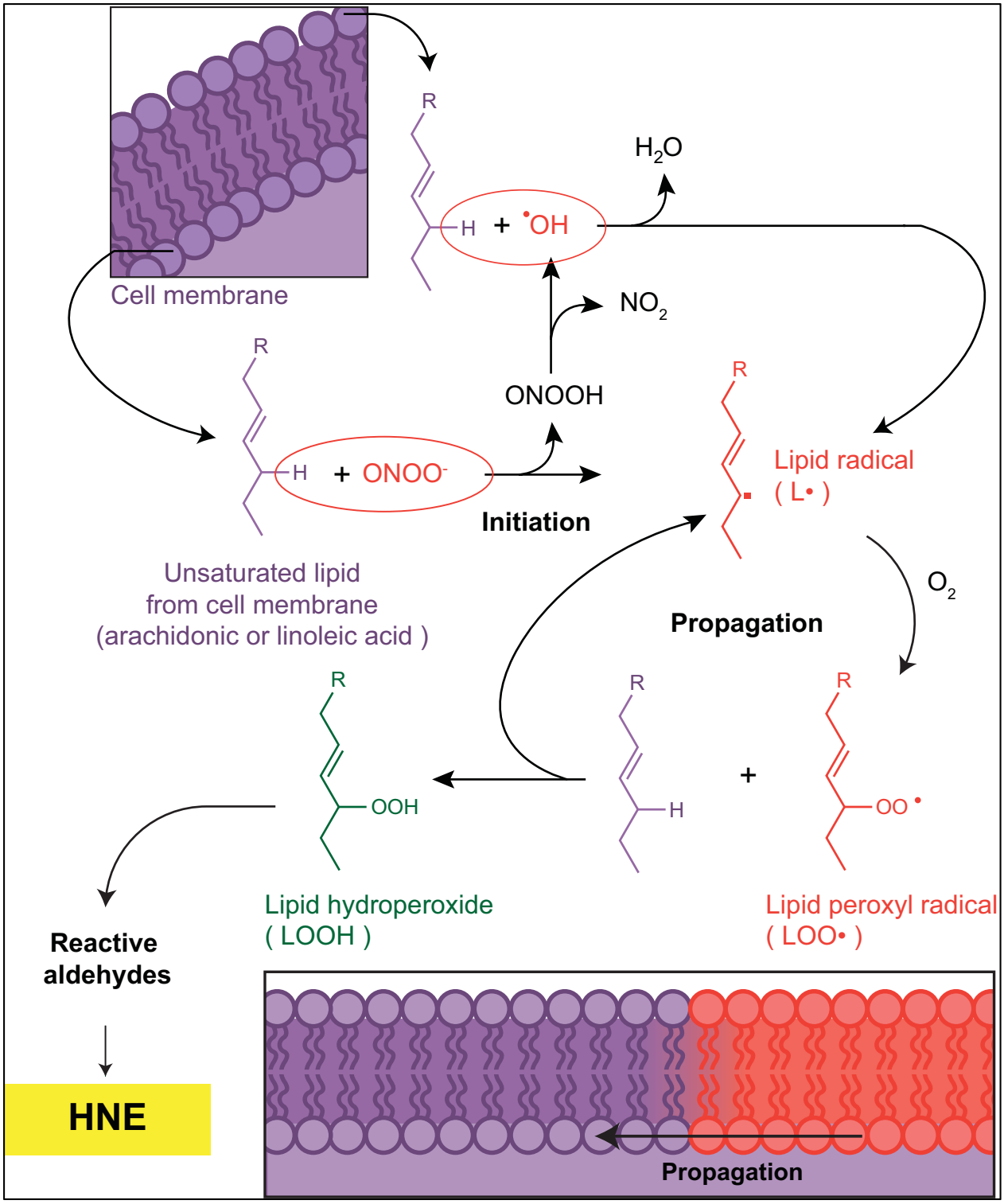


Figure 12. Lipid peroxidation

Lipid peroxidation is a chain reaction in which a reactive oxygen (or nitrogen) species react with unsaturated lipids of the cell membrane and produce reactive aldehydes (adapted from Tim Vickers/ Wikimedia Commons / CC-BY-SA-3.0).

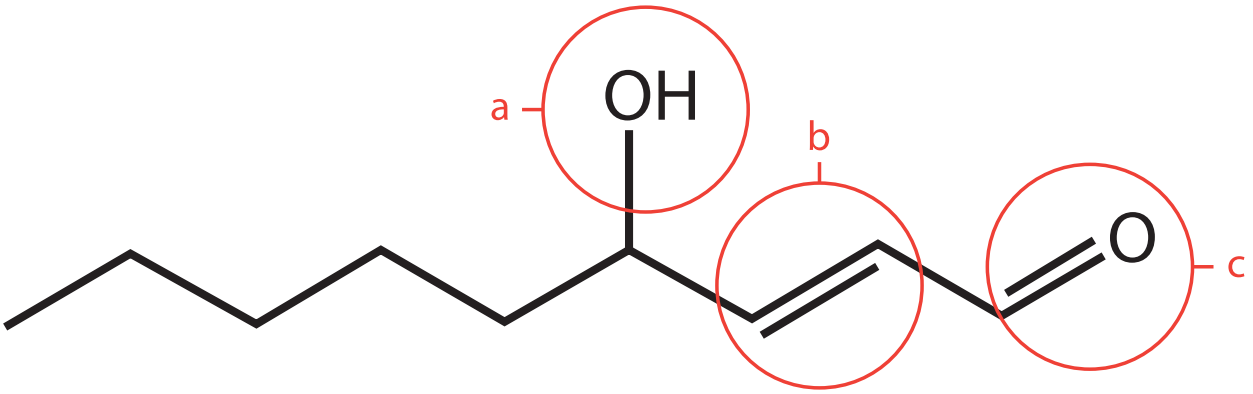


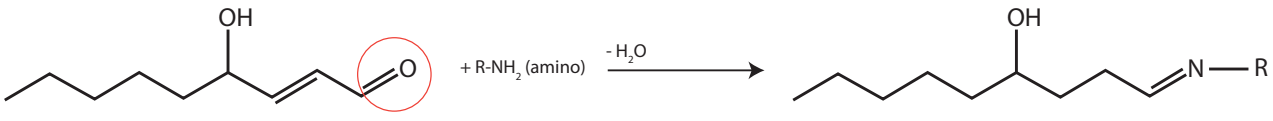
Figure 13. 4-Hydroxynonenal (HNE) molecule

The HNE molecule has three functional groups : **a.** a hydroxyl group (OH), **b.** a CC double bond and **c.** CO carbonyl group(Poli et al. 2008).

1.4.2 HNE adducts and target proteins

Important reactions for HNE's biochemical effects, as well as its metabolism, are Schiff base formation and Michael additions (see Figure 14) (Schaur 2003). Interaction between HNE and cell proteins has often been described; many HNE-binding proteins have been found, including heat shock proteins 72 and 90 (Hsp-72 & Hsp-90), CytoC, Ca^{2+} ATPase, albumin and many more (Carbone et al. 2004; Carbone et al. 2005; Isom et al. 2004; Akaishi et al. 2004; Aldini et al. 2006; Moreau et al. 2005). In addition, growing evidence supports a role for HNE as a physiologic modulator of signal transduction and of posttranslational modification(Poli et al. 2008). Our laboratory has also shown HNE's ability to induce transcriptional and posttranscriptional modifications of MMP-13 and Coll II(Morquette et al. 2006).

a. Schiff base formation



b. Michael addition

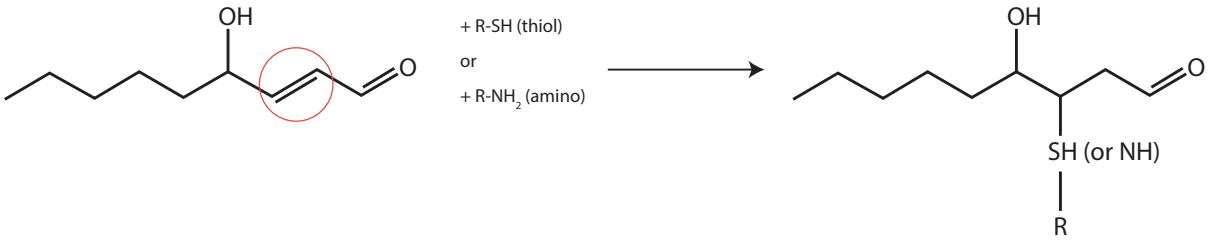


Figure 14. 4-Hydroxynonenal (HNE) common reactions

a. Formation of Schiff base is made possible by HNE's carbonyl base(Poli et al. 2008; Schaur 2003). **b.** Michael addition reaction between thiol or amino group and the CC double bond in HNE(Poli et al. 2008; Schaur 2003) (adapted from Poli and al. (2008). Fig 1 & 2).

HNE can be found in healthy human organs in small concentration but elevated HNE levels has been detected in a great number of human diseases ranging from Alzheimer's disease to Multiple sclerosis(Poli et al. 2008). Increased evidence suggests its significance in the pathogenesis of major human chronic diseases(Poli et al. 2008). Previous studies have linked HNE to OA by showing higher concentrations of HNE in chondrocytes and synovial fluid of OA patients when compared to healthy individuals(Morquette et al. 2006; Vaillancourt et al. 2007). In OA, HNE can be found in either protein-adduct form, such as with Coll II, or in free form (see Figure 15)(Morquette et al. 2006; Vaillancourt et al. 2007). It is thought that protein modification is the most likely one if not the main mechanism by which HNE can modulate physiological and pathophysiological processes(Poli et al. 2008).

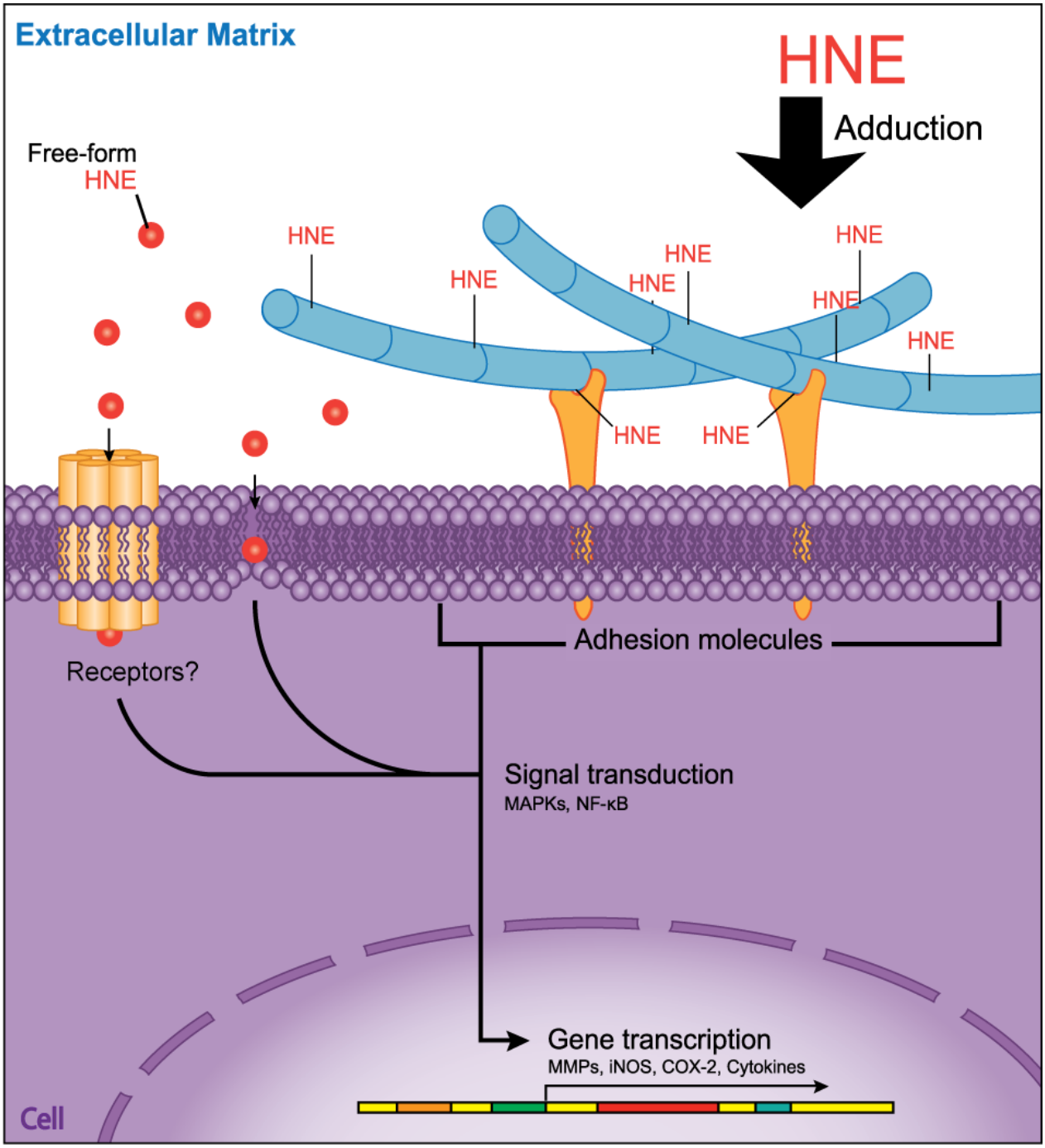


Figure 15. Effect of 4-Hydroxynonenal (HNE) signalling on cell function

Free-form and/or protein-bound HNE lead to activation of different signalling pathways therefore to production of cyclooxygenase-2 (COX-2), pro-inflammatory cytokines, metalloproteinases (MMPs), inducible nitric oxide synthase (iNOS), etc.

1.4.3 Metabolism of HNE

Because HNE is toxic and normally found, in low levels, in many human cells, there exist multiple pathways that can metabolize it. There are three main pathways the organism use to detoxify HNE: reduction by alcohol dehydrogenase or aldose reductase to 1,4-dihydroxynonene (DHN), oxidation by aldehyde dehydrogenases to form hydroxynonenoic acid (HNA) and conjugation with glutathione (GSH) by glutathione S-transferase (GST) (see Figure 16) (Poli et al. 2008; Pappa et al. 2003). GST isoenzyme GST4A-4 has a high specificity for HNE and plays an important role in protection against oxidative damage(Gallagher et al. 2006; Awasthi 2006; Vaillancourt et al. 2008). Another molecule that might be of significant importance in decreasing HNE toxicity is carnosine. Studies have shown it traps HNE by forming a stable covalent adduct thus inhibiting its ability to bind other proteins and activate metabolic pathways(El-Bikai et al. 2010; Poli et al. 2008). It has been suggested that carnosine might react with HNE by mimicking the preferred HNE addition sites in proteins(Aldini et al. 2002).

Though HNE is metabolized at a very rapid rate, it can still play an important role in protein modification as it activates a number of signaling pathways; more so when concentrations are elevated it as is the case in many pathologies(Poli et al. 2008). That is why novel ways to decrease HNE levels have and are being studied around the globe.

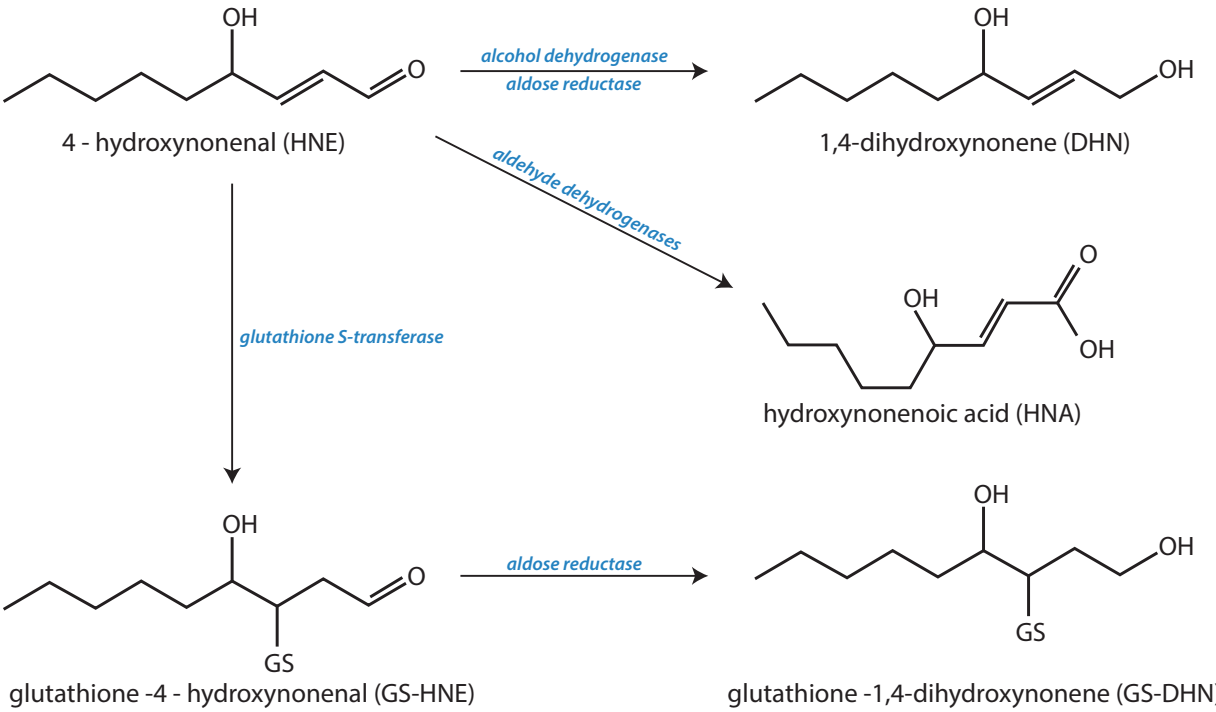


Figure 16. 4-Hydroxynonenal (HNE) metabolism

HNE's main metabolic pathways(Poli et al. 2008; Pappa et al. 2003) (adapted from Pappa and al. (2003) Scheme 1).

1.4.4 HNE and signalling pathways

It was long thought that HNE's biological and pathological effects where purely the result of protein modifications in cells and their environments. It has since been shown that HNE's role in pathophysiology is also greatly attributable to its ability to activate several signalling pathways(Esterbauer et al. 1991; Esterbauer et al. 1992; Benderdour et al. 2003; Morquette et al. 2006; Vaillancourt et al. 2007). Indeed, HNE has been linked to MAPK, JNKs and caspase pathways, to name only a few (Parola et al. 1998; Poli et al. 2008). These have many effects on cell activity as the pathways activated by HNE lead to cell stress and ultimately to apoptosis(Vaillancourt et al. 2008). At non-toxic levels, HNE can interact with histidine residues in JNKs which causes both their nuclear translocation and their activation(Parola et al. 1998). The activity and expression of key

inflammatory mediators are regulated by JNKs and p38 MAPK pathways through AP-1 activation(Herlaar & Brown 1999). These inflammation mediators include COX-2, cytokines such as TNF-α and IL-1β as well as proteases such as MMP-2, MMP-9 and MMP-13.

Also important is HNE's inhibitory effect on the NF-κB pathway. Some studies have shown that HNE binds to IκB blocking its phosphorylation and subsequently preventing NF-κB's translocation into the nuclei; greatly downregulating expression of NF-κB regulated genes(Leonarduzzi et al. 2004). In human OA chondrocytes, treatment with HNE appears to block selectively the NF-κB-regulated iNOS expression through IKKα inactivation by HNE/IKKα adducts formation(Vaillancourt et al. 2007). Poli and al. have suggested this might sustain inflammation and further lead to tissue degeneration by interfering with the immune response(Poli et al. 2008).

1.5 Inducible nitric oxide synthase (iNOS) inhibitors

To produce NO, NOS enzymes proceed to the oxidation of a nitrogen on the amino acid L-arginine (see Figure 17)(Fukuto & Chaudhuri 1995). This has led to research for NOS inhibitors using structural analogs of L-arginine since these analogs would compete with L-arginine binding to the enzyme(Furfine et al. 1993; Moore & Webber 1994; Misko et al. 1993; Marletta 1994).

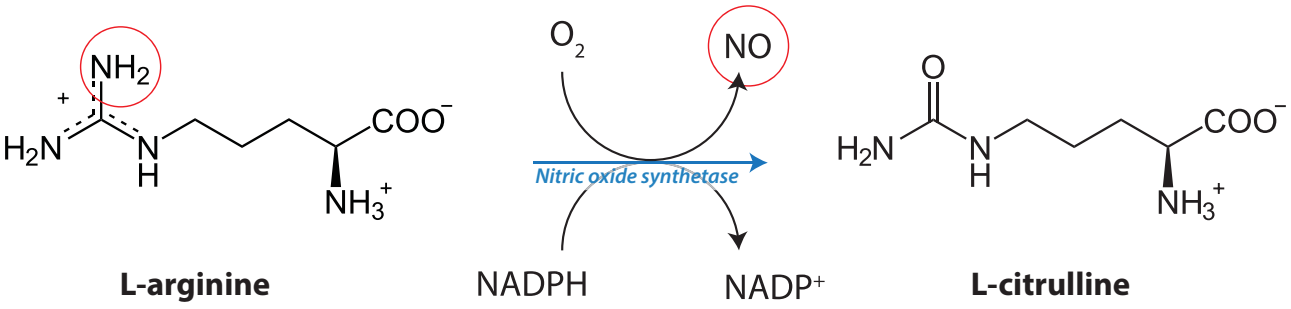


Figure 17. Nitric oxide (NO) synthesis by nitric oxide synthase (NOS)

Oxidation of L-arginine into L-citrulline by nitric oxide synthase (NOS) to generate nitric oxide (NO) (adapted from Fukuto and al., 1995 and NEUROtiker/ Wikimedia Commons / CC-BY-SA-3.0)(Fukuto & Chaudhuri 1995).

1.5.1 L-N^6- (l-iminoethyl)lysine (L-NIL)

Some of the analogs tested were N^G-monomethyl-L-arginine (L-NMA), N^G-nitro-L-arginine (L-NNA) and L-N^6-(l-iminoethyl)lysine (L-NIL) (see Figure 18)(Furfine et al. 1993; Moore & Webber 1994). L-NMA and L-NNA both inhibited NO synthesis but their use showed a marked and sustained increase in blood pressure indicating they were inhibiting the constitutive version of NOS(Rees 1989; Marletta 1994). Since NO plays an important role in normal physiology, it is crucial that a NOS inhibitor be specific to the inducible form in order to have potential clinical use against pathological overproduction of NO(Moore & Webber 1994). L-NIL was shown to have great selectivity for iNOS because of differences in the L-arginine binding sites of the different forms of NOS(Moore & Webber 1994). Some researchers have taken advantage of L-NIL specificity to study the role of iNOS in the pathophysiology/pathogenesis of OA both *in vitro* and *in vivo*(Pelletier et al. 1998; Pelletier et al. 1999; Pelletier et al. 2000; Walker et al. 2000; Hansel et al. 2003). Used on an experimental canine OA model, oral administration of L-NIL has attenuated OA progression and reduced cartilage matrix brakedown(Pelletier et al. 1998).

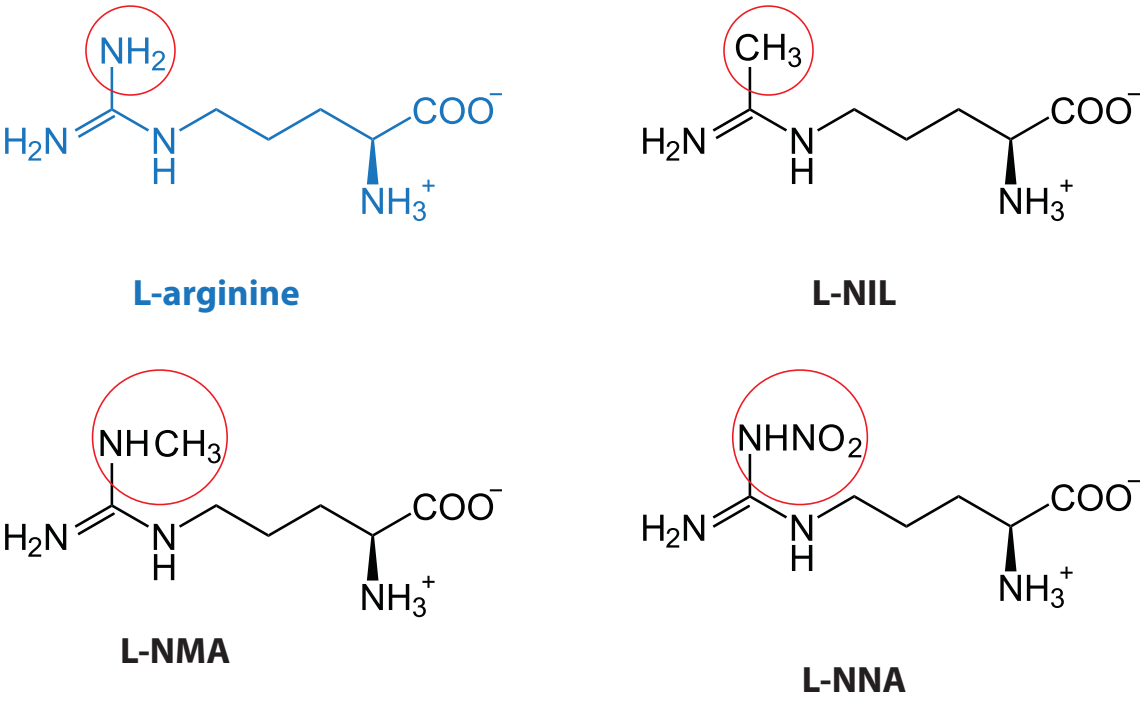


Figure 18. L-arginine analogs

Molecular structures of L-arginine, N^G-monomethyl-L-arginine (L-NMA), N^G-nitro-L-arginine (L-NNA) and L-N^6- (l-iminoethyl)lysine (L-NIL) (adapted from Moore and al., 1994 and NEUROtiker/ Wikimedia Commons / CC-BY-SA-3.0)(Moore & Webber 1994).

1.6 Objectives and hypothesis

Our lab has been investigating HNEs the pathophysiological role of HNE in OA for a few years now. Our research group was the first to report the involvement of HNE in the degradation and destruction of cartilage in OA through its ability to induce transcriptional and posttranslational modifications of Coll II and MMP-13, its capacity to induce cell death as well as its selective upregulation of COX_2 expression(Morquette et al. 2006; Vaillancourt et al. 2007; Vaillancourt et al. 2008). We have studied it both *in vitro* and *in vivo* and have proven its importance in the pathogenesis of OA(Morquette et al. 2006; Shi et al. 2014; El-Bikai et al. 2010).

Because NO plays such a crucial role in LPO through both its derivative $ONOO^-$ and its ability to generate ROS through NOX activation and since its main source in OA tissues is iNOS, we hypothesized that selectively inhibiting iNOS would protect against LPO, more specifically, that it would reduce HNE production.

Therefore, the aim of the present study was to investigate the ability of iNOS inhibitor, L-NIL, to prevent LPO product generation through NO inhibition in human OA chondrocytes.

CHAPTER 2: Article

Inhibition of inducible nitric oxide synthase prevents lipid peroxidation in osteoarthritic chondrocytes

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Key word: Nitric oxide, Lipid peroxidation, 4-Hydroxynonenal, Osteoarthritis, Cartilage, Glutathione-s-transferase

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Author contribution :

Mireille Bentz:

Experimentation, statistical analysis, contribution to result interpretation and paper preparation

Charlotte Zaouter and *Qin Shi:*

Assistance in certain experiments and in extraction of chondrocytes from human cartilage

Hassan Fahmi, Florina Moldovan and *Julio C. Fernandes:*

Data evaluation and interpretation and paper preperation

Mohamed Benderdour:

Study design and supervision, data evaluation and interpretation and paper preperation

All authors have approved final version of paper.

2.1 Abstract

Nitric oxide (NO) and the lipid peroxidation (LPO) product 4-hydroxynonenal (HNE) are considered to be key mediators of cartilage destruction in osteoarthritis (OA). NO is also known to be an important intermediary in LPO initiation through peroxynitrite formation. The aim of the present study was to assess the ability of the inducible NO synthase (iNOS) inhibitor N-iminoethyl-L-lysine (L-NIL) to prevent HNE generation via NO suppression in human OA chondrocytes and cartilage explants. Human OA chondrocytes and cartilage explants were treated with L-NIL and thereafter with or without interleukin-1beta (IL-1β) or HNE at cytotoxic or non-cytotoxic concentrations. Parameters related to oxidative stress, apoptosis, inflammation and catabolism were investigated. L-NIL stifled IL-1β-induced NO release, iNOS activity, nitrated proteins and HNE generation in a dose-dependent manner. It also blocked IL-1β-induced inactivation of the HNE-metabolizing glutathione-s-transferase (GST). L-NIL restored both HNE and GSTA4-4 levels in OA cartilage explants. Interestingly, it also abolished IL-1β-evoked reactive oxygen species (ROS) generation and p47 NADPH oxidase activation. Furthermore, L-NIL significantly attenuated cell death and markers of apoptosis elicited by exposure to a cytotoxic dose of HNE as well as the release of prostaglandin E_2 and metalloproteinase-13 induced by a non-cytotoxic dose of HNE. Altogether, our findings support a beneficial effect of L-NIL in OA by (i) preventing the LPO process and ROS production via NO-dependent and/or -independent mechanisms and (ii) attenuating HNE-induced cell death and different mediators of cartilage damage.

2.2 Introduction

Nitric oxide (NO) is a free radical that undergoes a variety of reactions in tissues with other radicals, such as superoxide or molecular oxygen, leading to the generation of highly-damaging nitrosating species [Squadrito and Pryor, 1998]. It has also been shown to cause nitrosation or oxidation of zinc finger-containing proteins involved in DNA repair [Sidorkina et al., 2003]. Furthermore, in vitro and in vivo cell exposure to NO from donor drugs can modulate the activity of mitochondrial enzymes, metalloproteinases (MMPs) and protein kinases via a nitrosylation process [Gu et al., 2002;Gu et al., 2010]. NO overproduction damages cellular components, including lipids, which results in declining physiological function and cell death. NO reaction with lipids generates lipid peroxidation (LPO), giving rise to 4-hydroxynonenal (HNE) [Morquette et al., 2006]. LPO initiation by NO in biological systems requires the generation of superoxide anion and hydroxyl radicals [Carrico et al., 2009;Szabo and Modis, 2010]. A mechanism of hydroxyl radical production, which has been proposed, involves the reaction of superoxide radical with NO, forming peroxynitrite. Experiments with pure peroxynitrite have shown that it can decompose spontaneously into hydroxyl radicals and initiate the oxidative modification of both lipids and proteins. In vivo, the main sources of NO and anion superoxide remain inducible NO synthase (iNOS) and NADPH oxidase (NOX), respectively [Montezano and Touyz, 2011]. Consistent with these data, the ability of peroxynitrite to evoke LPO necessitates perfect coordination between iNOS and NOX expression and activity. Thus, the development of inhibitors of both enzymes could have substantial benefits in the treatment of various disease conditions, including osteoarthritis (OA), in which oxidative stress is increased.

There is ample evidence that NO plays a key role in OA pathogenesis. Upon exposure to pro-inflammatory cytokines, OA cartilage produces excessive amounts of iNOS protein and its end-product NO [Amin et al., 1995;Castro et al., 2006]. High levels of nitrite/nitrate also have been found in synovial tissue from OA patients [McInnes et al.,

1996]. It has been hypothesized that NO contributes to the development of arthritic lesions by inhibiting the synthesis of cartilage matrix macromolecules [Amin and Abramson, 1998] and by inducing chondrocyte death [Nakagawa et al., 2010], which could further contribute to extracellular matrix (ECM) reduction in OA. For example, anterior cruciate ligament transection in rabbits provokes chondrocyte apoptosis and NO production, events that can, however, be prevented by treatment with iNOS inhibitors [Hashimoto et al., 1998]. The suppression of NO generation also significantly curbs interleukin-1beta (IL-1β)-induced chondrocyte apoptosis [Chen et al., 2006] and reduces the progression of cartilage damage in experimental OA model in dog [Pelletier et al., 1998].

Like NO and reactive oxygen species (ROS), aldehydes are electrophiles that bind to nucleophilic groups of proteins, but their relatively longer half-lives make them candidates for the propagation of damage to neighbouring cells [Uchida, 2003]. Aldehyde levels increase significantly under intense oxidative stress, and this elevation is believed to contribute to the development of many pathological conditions [Poli et al., 2008]. HNE, the principal α,β-unsaturated aldehyde formed from the LPO of both ω-3 and ω-6 polyunsaturated fatty acids, is of specific interest in the present study as its formation is enhanced in synovial fluid from OA patients [Morquette et al., 2006]. In addition, growing evidence supports its role as a key modulator of catabolic and inflammatory mediators known for their involvement in the OA process [Morquette et al., 2006;Vaillancourt et al., 2008;El-Bikai et al., 2010]. The electrophilic nature of HNE makes it highly reactive with specific amino acids in proteins via Michael-type addition: the sulfhydryl group of cysteine (CYS) or lipoic acid, the □-amino group of lysine (LYS), or the imidazole function of histidine (HIS) [Esterbauer et al., 1991;Wakita et al., 2011]. HNE homeostasis depends primarily on many factors, in addition to the magnitude of free radical production. Thus, conjugation to glutathione (GSH) by glutathione-s-transferase A4-4 (GSTA4-4) is a major route of HNE elimination [Balogh and Atkins, 2011]. It has been shown that GSTA4-4 metabolizes HNE with high catalytic efficiency

through its conjugation to GSH and has been suggested to be a major component of cellular defence against HNE toxicity [Sharma et al., 2011;Vaillancourt et al., 2008].

While investigating the potential regulation of HNE levels in OA cartilage, we obtained data indicating that its production is regulated by NO generation. In this study, we tested the hypothesis that suppression of NO release with the iNOS inhibitor N-iminoethyl-L-lysine (L-NIL) is a novel strategy to abrogate the LPO process and HNE generation in OA.

2.3 Materials and Methods

Specimen selection and chondrocyte culture

Discarded human post-surgery OA articular cartilage was obtained from OA patients (n=21, age 67±9 years mean±SEM) who underwent total knee arthroplasty. Informed consent was received from them to use their tissues for research purposes. All patients were evaluated by rheumatologists who followed American College of Rheumatology criteria [Altman et al., 1986]. The experimental protocols and research into human tissues were approved by the Research Ethics Board of Hôpital du Sacré-Cœur de Montréal.

OA knee cartilage specimens were sliced and rinsed before chondrocyte extraction by sequential enzymatic digestion, as described previously [Morquette et al., 2006]. Cartilage samples were digested with 1 mg/ml of pronase (Sigma-Aldrich, Oakville, ON, Canada) for 1 h at 37ºC, and then with 2 mg/ml of type IV collagenase (Sigma-Aldrich) for 6 h in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 units/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen). Chondrocytes 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 experimentation.

Experimental culture conditions

OA chondrocytes ($2x10^5$ cells/cm^2) were pre-treated with increasing concentrations of L-NIL (0-20 µM, Pfizer Canada Inc., Kirkland, QC, Canada) for 1 h, followed by incubation with or without either 1 ng/ml of IL-1β (Sigma-Aldrich), 10 µM 3-morpholinosydnonimine (SIN, Sigma-Aldrich) or cytotoxic (20 µM) or non-cytotoxic (10 µM) doses of HNE (Cayman Chemical, Ann Arbor, Ml, USA) for 24 h in 1% FBS-DMEM.

Prostaglandin E2 (PGE_2), metalloproteinase-13 (MMP-13) and NO determination

After chondrocyte incubation for 24 h, the medium was collected, and PGE_2 and MMP-13 levels were assessed by enzyme immunoassay (Cayman Chemical) and with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), respectively. Detection sensitivity was 9 and 8 pg/ml, respectively. All assays were performed in duplicate. Nitrite, a stable end-product of NO, was quantified in the supernatant according to a spectrophotometric method based on Griess reaction [Green et al., 1982]. Absorbance was measured with a micro-ELISA Vmax photometer (Bio-Tek Instruments, Winooski, VT, USA).

Cell viability

Chondrocyte viability was evaluated, as described previously [Mosmann, 1983], by 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) assay in 96-well plates (Fisher Scientific Company, Ottawa, ON, Canada) by incubating the cells with 0.5 mg/ml MTT reagent (Sigma-Aldrich) for 15 min at 37°C. Then, 100 µl of solubilization solution (0.04 M HCl-isopropanol) was added, formazan salt was dissolved, and absorbance was read at 570 nm with the micro-ELISA Vmax photometer (Bio-Tek Instruments).

iNOS activity assay

Culture supernatants were removed and the cells were washed 3 times with phosphate-buffered saline (PBS) and lysed by incubation with lysis buffer (40 mM Tris (pH 8.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 0.3 mM Na_3VO_4, 1 mM dithiothreitol) supplemented with protease inhibitor cocktail. Protein concentration of the cell lysates was measured by Bradford protein

assay (Bio-Rad Laboratories, Mississauga, ON, Canada). iNOS activity was assessed, as described previously [Sosroseno et al., 2011]. Briefly, 25 µg of chondrocyte lysate were incubated for 2 h at 37ºC in 100 µl of 20 mM Tris-HCl (pH 7.9) containing 5 µM tetrahydrobiopterin (BH_4), 5 µM FAD, 1 mM dithiothreitol, 1 mM NADPH and 1 mM L-arginine. All materials were purchased from Sigma-Aldrich. The reaction was then stopped by adding lactate dehydrogenase (10 U/ml), and NO levels were measured by the Griess method described above. Each assessment was repeated 3 times.

Protein detection by Western blotting

20 µg of total proteins of chondrocyte lysates, treated under the indicated conditions, were loaded for discontinuous 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They were then transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories) for protein immunodetection and semi-quantitative measurement [Morquette et al., 2006]. The primary antibodies deployed were rabbit anti-human NOX component p47phox (p47 NOX, Cell Signalling Technology, Inc., Danvers, MA, USA), anti-HNE (Cayman Chemical), anti-nitrotyrosine (Cayman Chemical), anti-caspase-3 (EMD Biosciences, Inc., San Diego, CA, USA), and anti-human β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After serial washes, the primary antibodies were revealed by goat anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Cell Signalling Technology, Inc.). Immunoreactive proteins were detected with SuperSignal blotting substrate (Pierce, Rockford, IL, USA) and exposed to Kodak X-Omat film (Eastman Kodak Company, Rochester, NY, USA).

Cellular level of HNE-protein adducts

Total cellular levels of HNE-protein adducts were calculated in chondrocyte extracts under the indicated conditions by in-house ELISA [Morquette et al., 2006]. HNE-modified bovine serum albumin (BSA) served as standard for HNE-protein adduct assay.

Immunohistochemistry

Human OA cartilage explants (~150 mg) were incubated for 1 h in the presence or absence of 10 mM L-NIL, followed by another incubation for 48 h in the presence of 1 ng/ml IL-1β. The cartilage specimens were fixed in TissuFix #2 (Laboratoires Gilles Chaput, Montreal, QC, Canada) for 24 h, then embedded in paraffin. Sections (5 μm) of paraffin-embedded specimens were de-paraffinized in toluene, rehydrated in a reverse-graded series of ethanol and pre-incubated with 0.25 units/ml chondroitinase ABC (Sigma-Aldrich) in PBS (pH 8.0) for 60 min at 37°C. The sections were subsequently washed in PBS, incubated in 0.3% Triton X-100 for 20 min, then placed in 3% hydrogen peroxide/PBS for 15 min. The slides were further incubated with blocking serum (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) for 60 min, after which they were blotted and overlaid with the primary antibody against HNE (1/200 dilution, EMD Chemicals, Inc. Gibbstown, USA) or GSTA4-4 (1/500 dilution, Sigma-Aldrich) for 18 h at 4°C in a humidified chamber.

Each slide was washed 3 times in PBS (pH 7.4), stained by the avidin-biotin complex method (Vectastain ABC kit), and incubated in the presence of biotin-conjugated secondary antibody for 45 min at room temperature, followed by the addition of avidin-biotin-peroxidase complex for 45 min. All incubations were undertaken in a humidified chamber at room temperature, and staining was developed with 3,3'-diaminobenzidine (DAKO Diagnostics Canada Inc., Mississauga, ON, Canada) containing hydrogen peroxide. The slides were counterstained with hematoxylin/eosin.

The sections were examined and photographed with a light Leica DM IRB microscope (Leica, Heerbrugg, Switzerland) equipped with a Leica DFC425 C camera (Leica). The presence of the antigen in cartilage was quantified by determining the number of chondrocytes that stained positive throughout cartilage thickness. Three sections from cartilage explants were examined, and the results were the means of positive chondrocytes counted in 3 light microscopic fields. The total number of chondrocytes and those staining positive in each field for the specific antigen were quantified. The final results were expressed as percentages of chondrocytes staining positive for the antigen (cell score), with the maximum score being 100%. Each slide was viewed by 2 independent readers who were blinded to treatment group allocation.

ROS measurement

Intracellular ROS formation was measured with MitoSOX™ Red reagent (Invitrogen). Oxidation of MitoSOX™ Red reagent by superoxide produces red fluorescence, as described by Zhou et al. [Zhou et al., 2011]. Briefly, chondrocytes were seeded at a density of 2×10^4 cells/well in 96-well black plates (Becton Dickinson, San Jose, CA, USA). They were pre-treated with increasing concentrations of L-NIL 1 h before their exposure to IL-1β. Fluorescence was measured with a fluorescence plate reader at 510-nm absorption and 580-nm emission, and the data were expressed as relative MitoSOX™ Red fluorescence. To detect ROS in cells, the chondrocytes were transferred to 8-well culture chamber slides (Lab-Tek, Nalge Nunc International, Naperville, IL, USA), and incubated with MitoSOX™ Red reagent and 4',6-Diamidino-2-phenylindole (DAPI). ROS generation was then measured every 30 s for 15 min with a Leica DM IRB fluorescence microscope (Leica) equipped with a Leica DFC425 C camera (Leica).

Total GST activity

Total GST activity was assessed with a commercial kit (Sigma-Aldrich) in 50 µg of chondrocyte extract. Briefly, the cells were homogenized on ice in 1 ml of buffer containing 180 mM KCl, 5 mM MOPS, and 2 mM EDTA, pH 7, and centrifuged for 10 min at 800 ×g at 4°C. The supernatants were subjected to enzyme assays after 10-min centrifugation at 6,000 ×g at 4°C. Protein levels were measured with a kit (Bio-Rad Laboratories) with BSA (Sigma-Aldrich) as standard. Activities were expressed in units/mg of proteins, where 1 unit was defined as the amount of enzyme catalyzing the conversion of 1 µmol substrate/min at 37°C.

DNA fragmentation

Cytoplasmic histone-associated DNA fragments were quantified with Cell Death Detection ELISA^{PLUS} kits (Roche Applied Science, Laval, QC, Canada) according to the manufacturer's recommendations. Briefly, cultured chondrocytes ($1x10^6$ cells) were lysed with lysis buffer for 30 min and centrifuged at 200 g for 10 min. The supernatant and a mixture of anti-histone-biotin and anti-DNA-peroxidase were added to streptavidin-coated microplates and incubated for 2 h at room temperature. Absorbance was measured at 405 nm after inclusion of the substrate.

Statistical analysis

All values are expressed as means±SEM unless indicated otherwise. Multiple comparisons were made by 1-way ANOVA, as required, followed by the Bonferroni multiple-comparison post-test. Statistical comparisons were performed with GraphPad Prism software, version 4b (GraphPad Software, San Diego, CA, USA). In all tests, the criterion for statistical significance was $p<0.05$.

2.4 Results

L-NIL prevents NO and iNOS production in OA chondrocytes

In our first objective, we performed initial experiments to demonstrate that L-NIL prevents NO release and inhibits iNOS activity in isolated human OA chondrocytes. Cells were treated for 1 h with increasing doses of L-NIL (0–20 μM) and thereafter with or without 1 ng/ml IL-1β for 24 h. The addition of L-NIL significantly reduced IL-1β -induced NO release (Fig. 1A) and iNOS activity (Fig. 1B) in a dose-dependent manner. At 10 μM, L-NIL suppressed NO release by 80% ($P<0.001$) and iNOS activity by 90% ($P<0.001$). Collectively, these data confirm that L-NIL is a potent iNOS inhibitor.

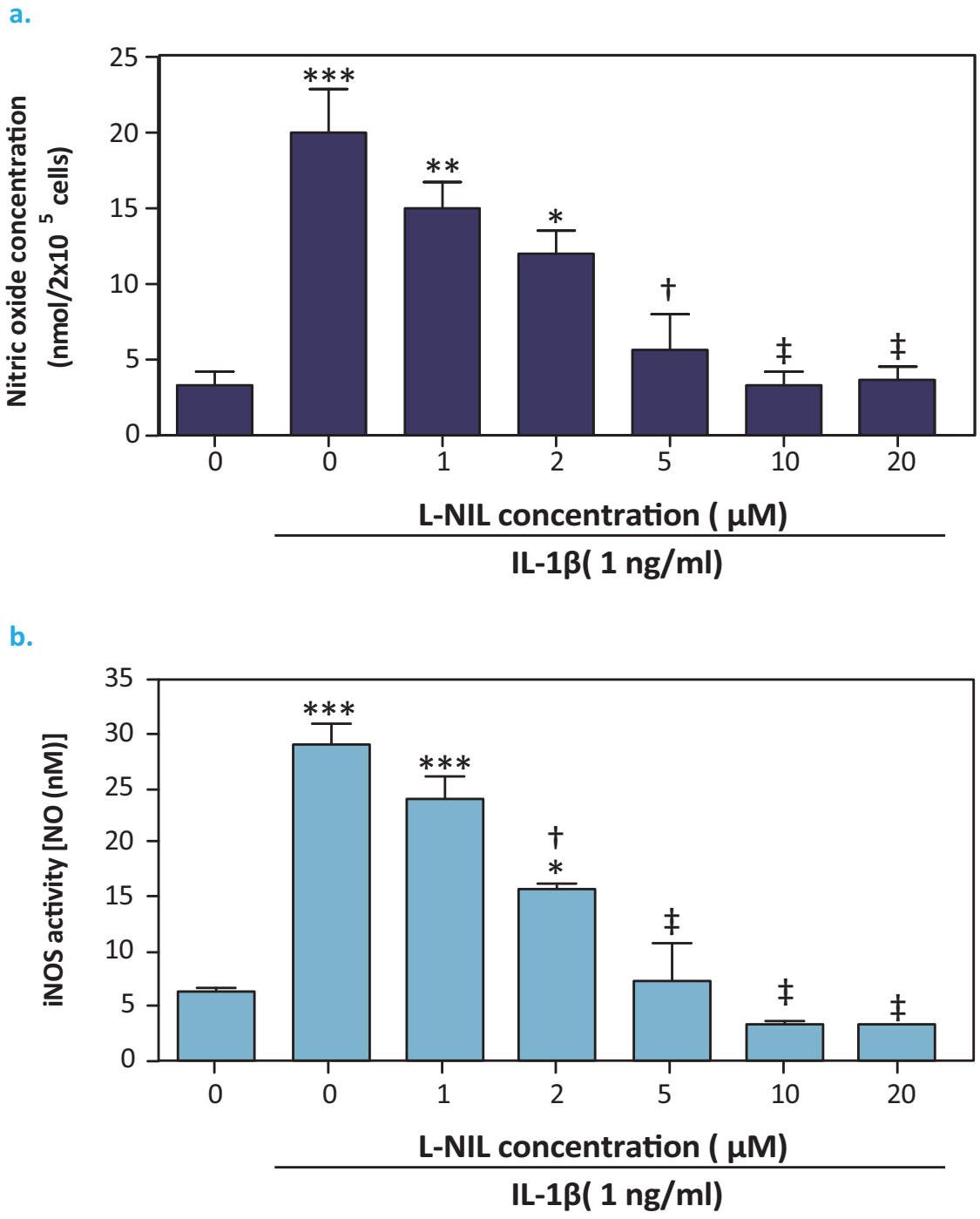

Fig. 1. N-iminoethyl-L-lysine (L-NIL) reduces nitric oxide (NO) release and inducible nitric oxide synthase (iNOS) activity. Human osteoarthritis (OA) chondrocytes were treated with increasing L-NIL concentrations (0–20 µM) for 1 h before incubation for 24 h in the presence of 1 ng/ml interleukin-1beta (IL-1β). **a.** NO release was assessed by the improved Griess method. **b.** iNOS activity was measured in the presence of arginine, BH4, and NADPH. Values represent the means_SEM of 3–4 separate experiments performed in duplicate. *P <0.05,**P <0.01,***P <0.001 compared to untreated cells (control) (1% FBS); †P <0.01, ‡P <0.001 compared to IL-1b-treated cells.

L-NIL blocks HNE production through NO and peroxynitrite inhibition

We and other research groups have reported the ability of NO to induce LPO via peroxynitrite generation [Morquette et al., 2006]. In the present study, we tested the hypothesis that NO inhibition suppresses peroxynitrite and, consequently, the production of HNE, a very reactive product of LPO. A previous study has shown that selective iNOS inhibition leads to reduction of HNE generation in renal cells (Noiri et al. 2001). As illustrated in Figure 2A, the addition of 1 ng/ml IL-1β or 10 μM SIN, a donor of free radicals, induced a similar pattern of both NO release and HNE-protein adducts production. Compared to untreated cells, NO levels reached 15x2.5 nmol/2x10^5 cells ($P<0.01$) and 12x3 nmol/2x10^5 cells ($P<0.05$), and HNE attained 600±55 and 650±86 pg/mg proteins ($P<0.01$) in the presence of SIN or IL-1β, respectively. Before testing the ability of L-NIL to inhibit HNE production, we investigated whether this drug blocks peroxynitrite generation. As expected, western blotting revealed that nitrotyrosine levels in nitrated proteins were lower in L-NIL-treated cells than in IL-1β treated cells (Fig. 2B). Interestingly, the addition of L-NIL at different dose levels also abolished IL-1β-induced HNE production. Our ELISA and western blotting data (Fig. 2C,D) disclosed that L-NIL, at 10 μM, prevented HNE generation ($P<0.01$) in cell extracts from IL-1β -treated OA chondrocytes. Afterwards, we tested the ability of L-NIL to prevent (or not) the IL-1 β -induced inactivation of HNEmetabolizing GST. As illustrated in Figure 2E, L-NIL addition to cultured cells blocked GST inactivation by IL-1β ($P<0.05$). Taken together, these findings strongly suggest that HNE production is linked, at least in part, to NO release and iNOS activation. L-NIL could be considered an interesting tool to abrogate both NO generation and LPO in OA chondrocytes. It also prevented impairment of redox status in IL-1β -treated cells.

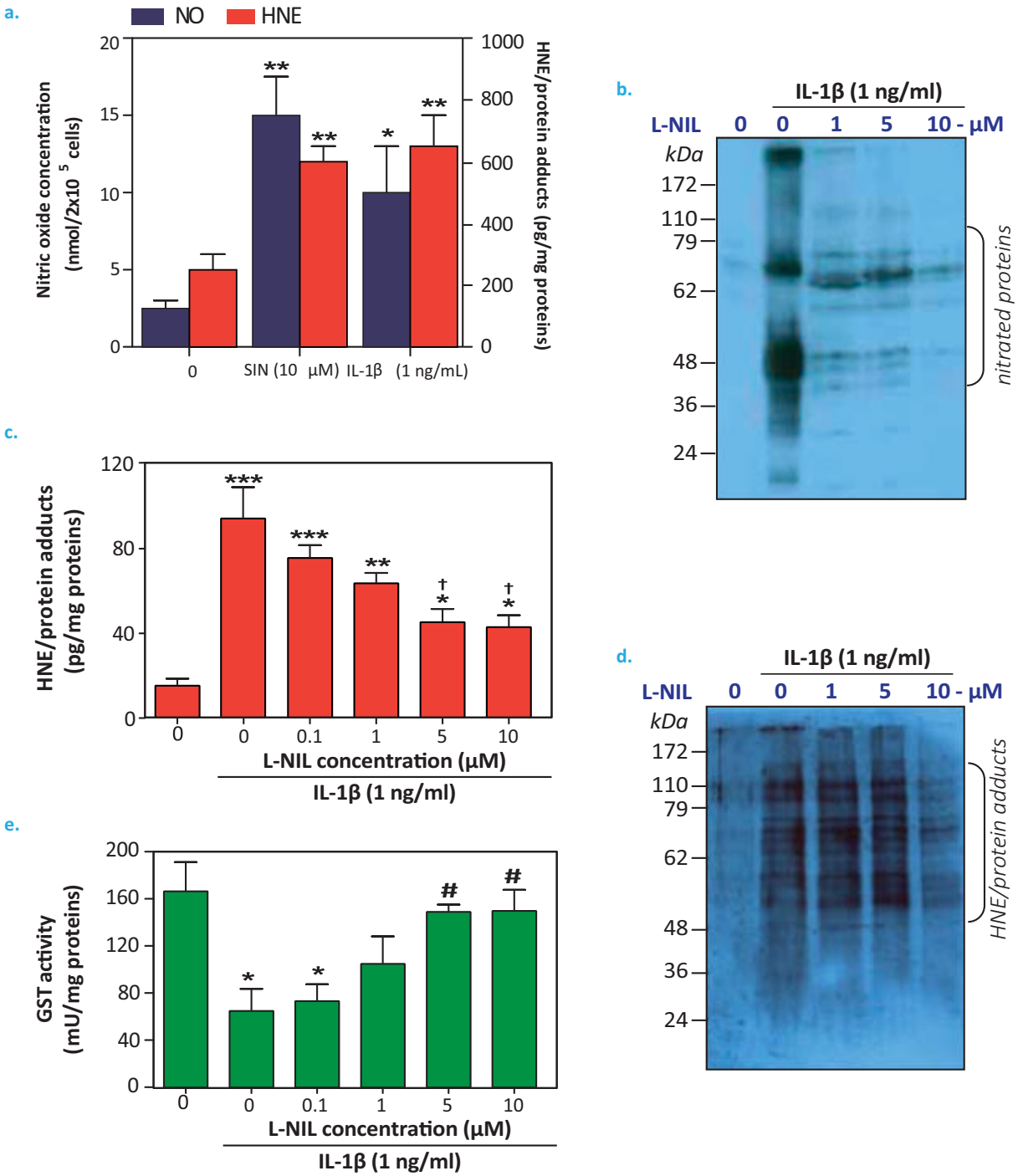

Fig. 2. N-iminoethyl-l-lysine (L-NIL) suppresses the lipid peroxidation product 4-hydroxynonenal (HNE). a: Confluent human osteoarthritic (OA) chondrocytes were treated with either 10 µM SIN (a NO donor) or 1 ng/ml IL-1β for 24 h. NO and HNE generation was assessed by the improved Griess method or ELISA, respectively. (**b-e)** Human OA chondrocytes were treated with increasing doses of L-NIL (0–10 µM) for 1 h, followed by incubation for 24 h in the presence or absence of 1 ng/ml IL-1b. The cells were then lysed and analyzed. Nitrated protein levels were evaluated by western blotting (**b)**, HNE/protein adducts were quantified by ELISA (**c)** and analyzed by western blotting (**d)**, and GST activity was assessed with a commercial kit (**e)**. The NO, HNE/protein adduct and GST activity results are expressed as nmol/2x10^5 cells, pg/mg proteins or mU/mg proteins, respectively, and represent the means_SEM of four independent experiments performed in duplicate.*P <0.05,**P <0.01,***P <0.001 compared to untreated cells (1% FBS); #P <0.05, †P <0.01 compared to IL-1b-treated cells alone.

Immunohistochemistry of HNE and GSTA4-4

Chondrocytes staining positive for HNE and GSTA4-4 were found in OA cartilage. Compared to the controls (Fig. 3A), chondrocytes stained positive for increased HNE, which reached 61% (Fig. 3B, $P<0.01$) in IL-1β-treated OA cartilage. However, 10 μM L-NIL significantly prevented IL-1β -induced HNE production in OA cartilage (Fig. 3C, $P<0.05$). Furthermore, compared to the controls (Fig. 3D), chondrocytes stained positive for decreased GSTA4-4, which reached 14% (Fig. 3E, $P<0.01$) in IL-1β-treated OA cartilage. However, 10 μM L-NIL significantly abolished IL-1β inhibition of GSTA4-4 expression in OA cartilage (Fig. 3F, $P<0.05$). Altogether, our results confirm that the iNOS inhibitor L-NIL attenuates oxidative stress and restores redox status by reducing HNE generation and reinstating GSTA4-4 expression in cartilage explants.

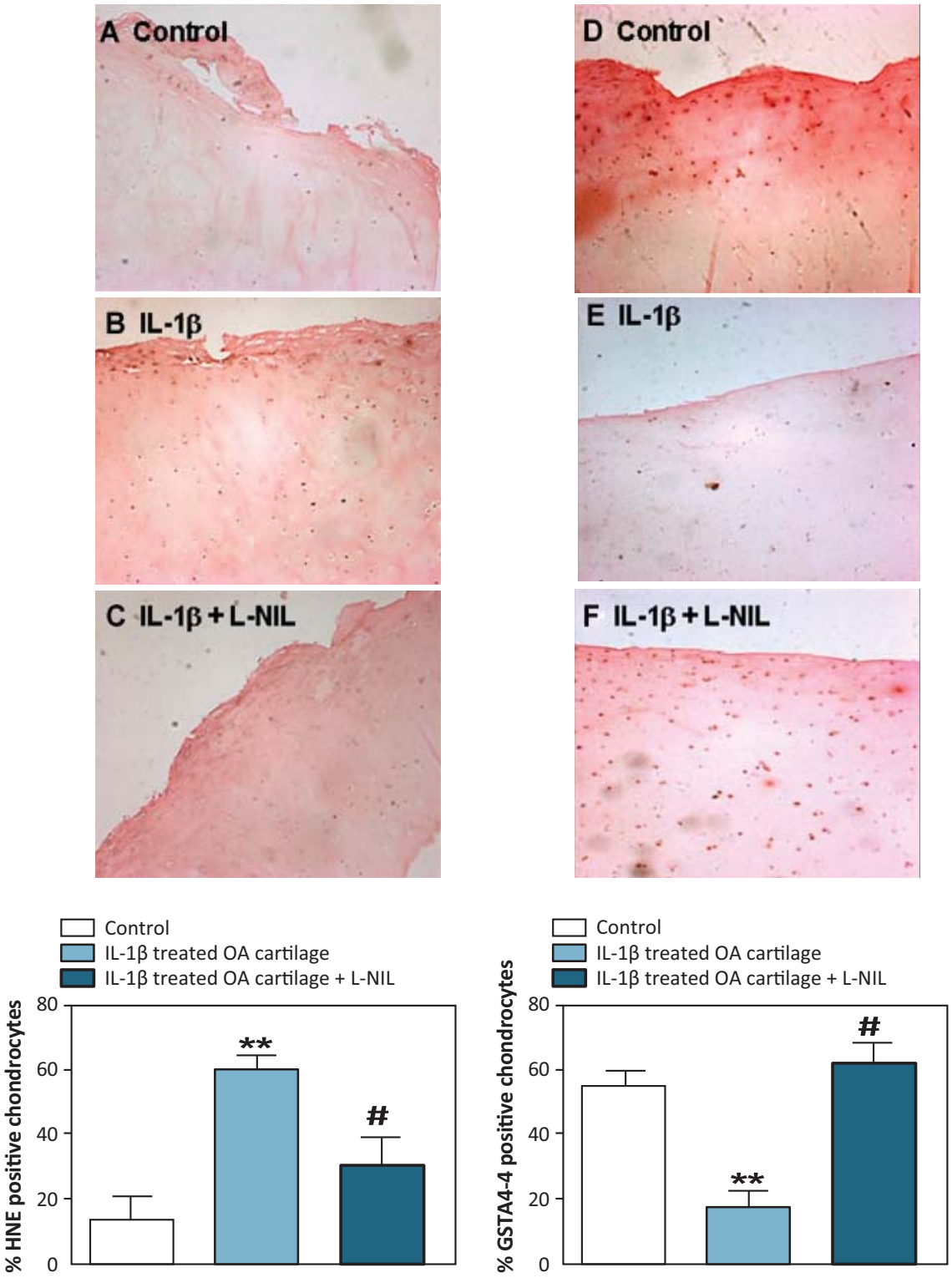

Fig. 3. **Immunohistochemistry of HNE and GSTA4-4. Representative section showing 4-hydroxynonenal (HNE)** (**A–C**) and GSTA4-4 (**D–F**) immunostaining in superficial zones of articular cartilage from human osteoarthritis (OA) cartilage explants treated or not for 1h with 10 µM L-NIL, followed by another incubation for 24 h with 1 ng/ml IL-1β (original magnification x100). The number of chondrocytes staining positive for HNE and GSTA4-4 were evaluated and analyzed as described in Materials and Methods section. **P <0.01 compared to untreated OA cartilage (control); #P <0.05 compared to IL-1β-treated cells.

L-NIL abolishes ROS generation and p47 NOX phosphorylation

It is well-documented that the generation of ROS, such as superoxide anion and hydroxyl radical, plays an important role in initiating LPO. Thus, the purpose of this part of the present study was to investigate whether L-NIL's capability to inhibit HNE production is attributed to its ability to also prevent ROS generation. To do so, OA chondrocytes were pre-treated with 10 μM L-NIL for 1 h, followed by exposure to 1 ng/ml IL-1β for 24 h. ROS generation was observed by fluorescence microscopy and then quantified with MitoSOX™ Red reagent. As illustrated in Figure 4B, elevated ROS levels were detected by fluorescence microscopy in OA chondrocytes treated with 1 ng/ml IL-1β compared to untreated cells (Fig. 4A). Interestingly, ROS production was quenched by L-NIL treatment (Fig. 4C). DAPI staining, which reveals nuclei, was employed to assess their number and to examine gross cell morphology. Quantitatively, our data indicated that relative MitoSOX™ Red fluorescence was higher in 1 ng/ml IL-1β-treated cells and reached 220±36 (P<0.05) (Fig. 4D). However, when the cells were treated with both 1 ng/ml IL-1β and 10 μM L-NIL, relative MitoSOX™ Red fluorescence decreased significantly to 121±16 (P<0.05) (Fig. 4D). Finally, an additional experiment was performed to determine the possible ability of L-NIL to inhibit p47 NOX phosphorylation, a ROS-generating enzyme. Western blotting analysis showed that 10 μM L-NIL prevented IL-1β-induced p47 NOX phosphorylation (Fig. 4E). Collectively, our data indicated that suppression of ROS production and ROS-generating NOX by the selective inhibitor of iNOS had a significant LPO outcome.

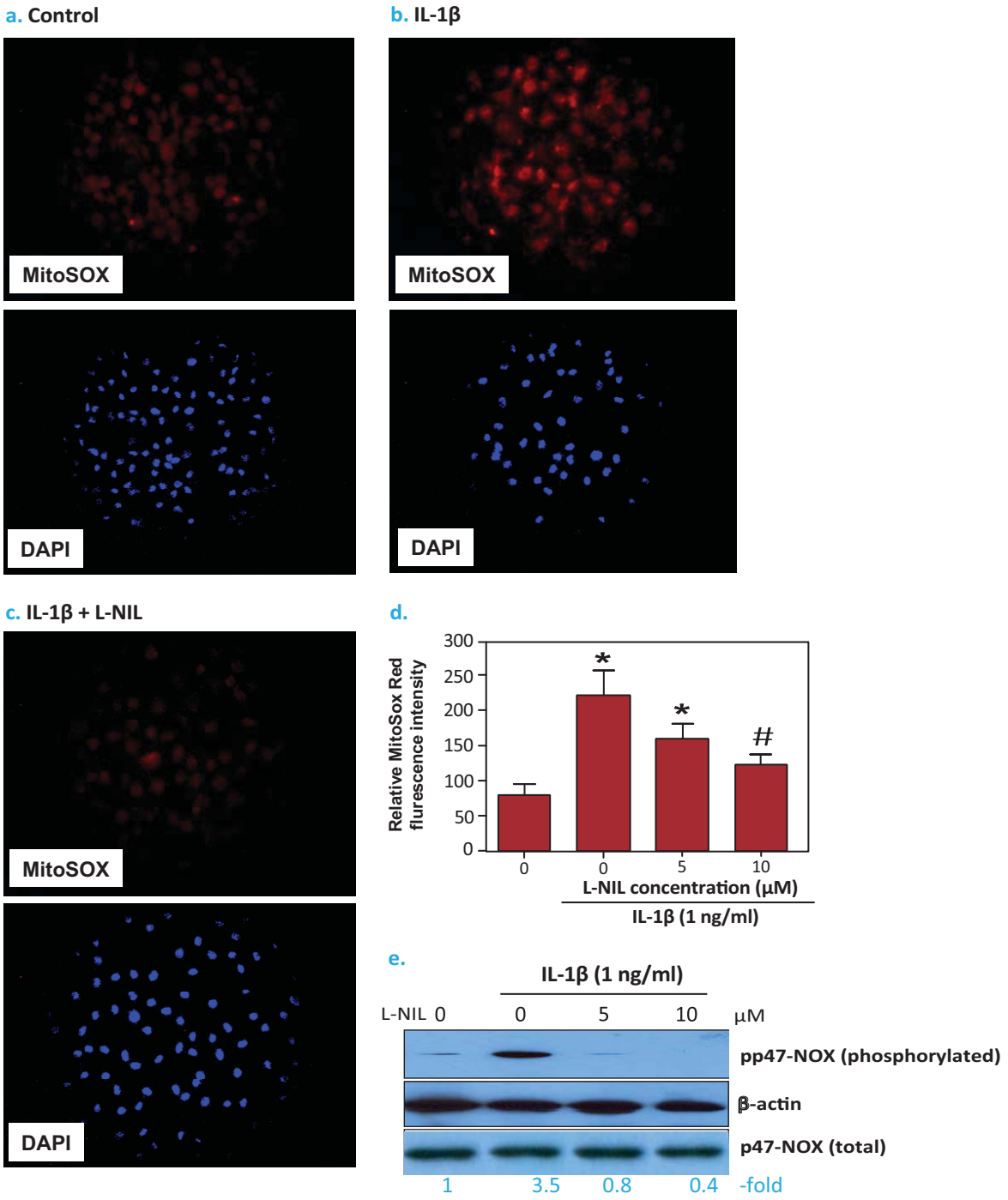

Fig 4. N-iminoethyl-L-lysine (L-NIL) suppresses reactive oxygen species (ROS) generation and NADPH oxydase (NOX) phosphorylation. (A-C) Confluent human osteoarthritic (OA) chondrocytes were treated for 1 h with 10 μM L-NIL, followed by incubation for 24 h in the presence or absence of 1 ng/ml IL-1β. ROS generation was revealed by fluorescence microscopy with MitoSOX™ Red reagent. (D & E) Cells were treated for 1 h with increasing concentrations of L-NIL (0-10 μM), followed by incubation for 24 h in the presence or absence of 1 ng/ml IL-1β. (D) ROS generation was quantified in cell extracts with MitoSOX™ Red reagent. ROS levels were expressed as relative MitoSOX™ Red fluorescence. (E) Total (p47) and phosphorylated (pp47) NOX protein were analyzed by Western blotting in protein extracts from human OA chondrocytes treated as described above. The data are means±SEM of 4 independent experiments. **P*<0.05 compared to untreated cells (control) (1% FBS); #*P*<0.05 compared to IL-1β-treated cells alone.

HNE-induced cell death is blocked by L-NIL

We reported that HNE up to 10 μM did not alter cell viability, but 20 μM HNE was cytotoxic and significantly decreased cell viability by approximately 50% [Vaillancourt et al., 2008]. We evaluated the ability of L-NIL to reduce HNE cytotoxicity in cultured chondrocytes. Cell viability was assessed with MMT reagent. After 24 h of incubation, pre-treatment with 5 and 10 μM L-NIL for 1 h before adding 20 μM HNE to the culture media prevented HNE-induced cell death (Fig. 5A) as well as markers of apoptosis, including caspase-3 activation (Fig. 5B) and DNA fragmentation (Fig. 5C). These data suggest that L-NIL probably prevents HNE's effects through direct HNE quenching.

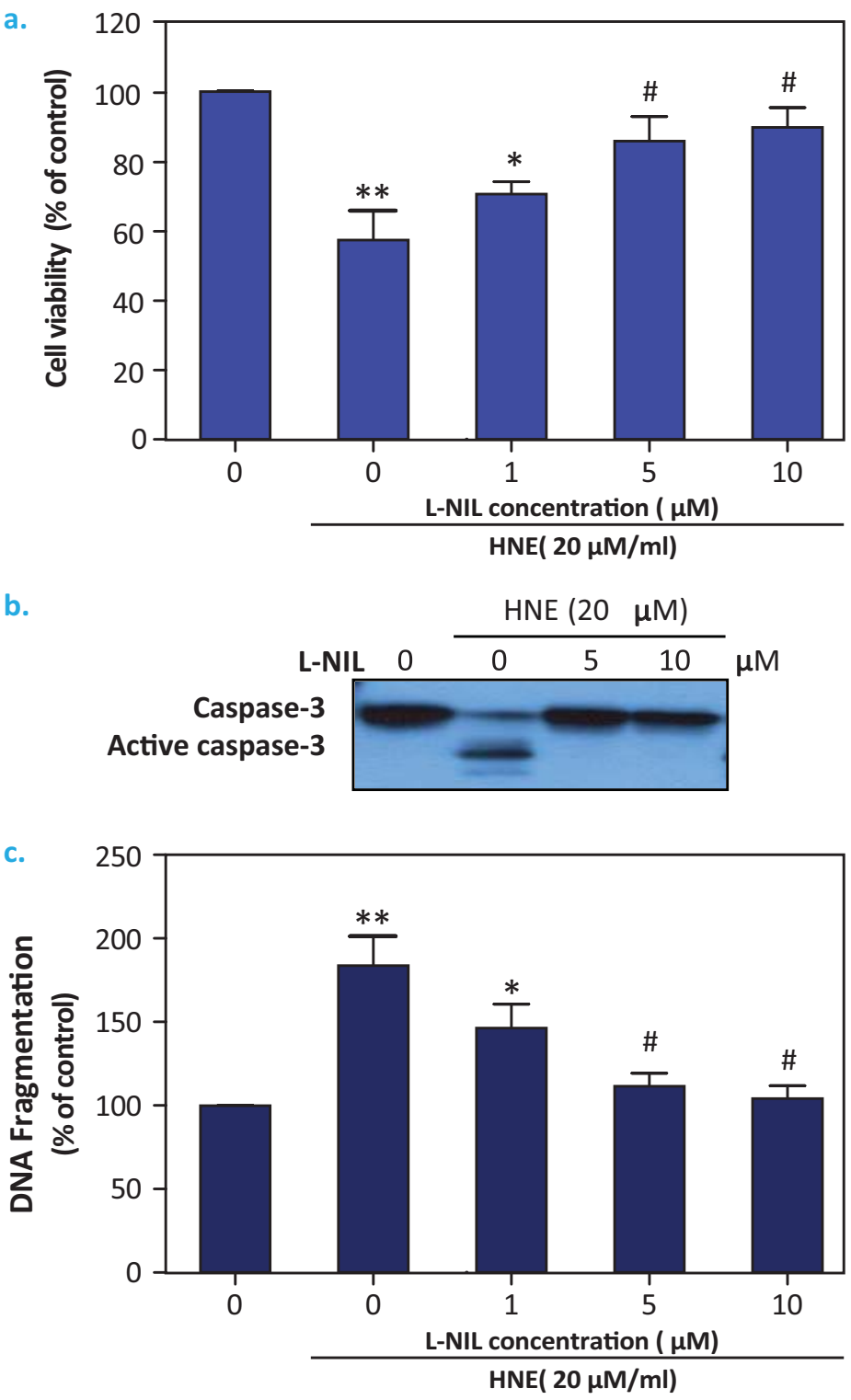

Fig 5. 4-Hydroxynonenal (HNE)-induced cell death is blocked by L-NIL. Chondrocytes were pre-incubated for 1 h with or without increasing doses of L-NIL (0-10 µM) followed by another incubation for 24 h with a cytototoxic HNE dose (20 µM). (A) Cell viability was evaluated by MTT assay. (B) Activation of caspase-3 was determined by Western blotting. (C) Cytoplasmic histone-associated DNA fragments were quantified with a kit. The data, expressed as % of untreated cells for cell viability and DNA fragmentation, are the means±SEM of 4 independent experiments. *P<0.05, **P<0.01 compared to untreated cells (1% FBS); $^{\#}P$<0.05 compared to HNE-treated cells alone.

HNE-induced PGE2 and MMP-13 production are abrogated by L-NIL

This part of our study was designed to verify L-NIL's ability to attenuate HNE-evoked the production of inflammatory and catabolic mediators known to be involved in cartilage damage, such as PGE_2 and MMP-13, respectively. When the cells were treated with non-cytotoxic doses of HNE (at 10 µM) [Vaillancourt et al., 2008], L-NIL prevented HNE-induced PGE_2 (Fig. 6A) and MMP-13 (Fig. 6B) release. At 10 µM, L-NIL reduced PGE_2 and MMP-13 levels by 55 (P<0.01) and 60% (P<0.05), respectively. Pro-MMP-13 is shown here because we have already previously shown both pro and active MMP-13 induction by HNE (Morquette et al. 2006). In fact, active MMP-13 showed a 2,5 fold increased when treated with 10 µM/ml of HNE when compared with untreated cells (Morquette et al. 2006).

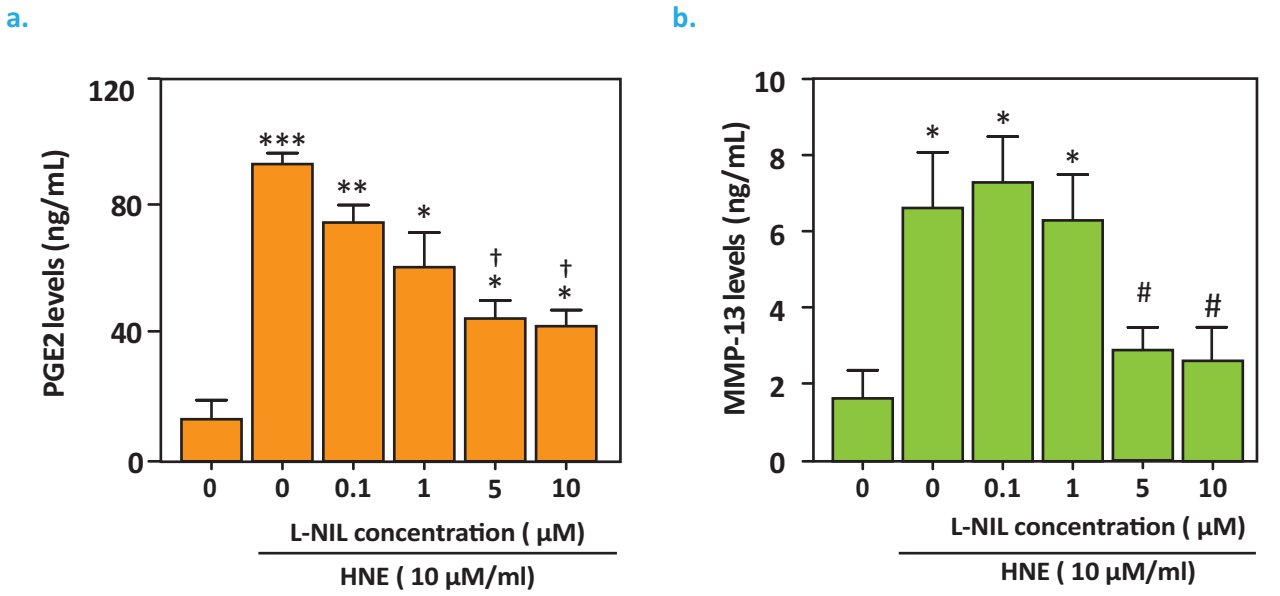

Figure 6: N-iminoethyl-L-lysine (L-NIL) prevents 4-hydroxynonenal (HNE) induction of prostaglandin E2 (PGE_2) and metalloproteinase-13 (MMP-13). Human osteoarthritic (OA) chondrocytes were treated for 1 h with different doses of L-NIL (1-10 µM) before incubation with non-cytotoxic (10 µM) doses of HNE for 24 h. Supernatants were collected, and PGE_2 (A) and MMP-13 (B) levels were measured with commercial enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) kits, respectively. The data, expressed as ng/ml for PGE_2 and MMP-13 protein, represent the means±SEM of 4 independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared to untreated cells (1% FBS); #P<0.05, †P<0.01 compared to HNE-treated cells alone.

2.5 Discussion

Articular cartilage is believed to be a major source of NO in OA joints and synovial fluid [Pelletier et al., 1998]. Previous *in vitro* studies have shown that chondrocytes produce NO as a key metabolite in response to stimuli such as mechanical stress, IL-1α, IL-1β and lipopolysaccharides (LPS) [Abramson, 2008]. In addition, iNOS is up-regulated in OA chondrocytes, resulting in excess NO and perpetuating the release of inflammatory cytokines and other catabolic processes. NO inhibits both proteoglycan and collagen synthesis, activates MMPs, mediates chondrocyte apoptosis and promotes chondrocyte inflammatory responses [Abramson, 2008]. These findings add to the prevailing hypothesis that NO is a pro-inflammatory and pro-apoptotic factor that, when present in excess, is detrimental to the joints and contributes to OA pathogenesis. A number of iNOS inhibitors have been developed to prevent NO production in OA joint tissues. Among them, L-NIL is a potent and selective iNOS inhibitor. However, the detrimental effects of NO in OA cartilage have been challenged by a number of reports showing that NO may inhibit pro-inflammatory responses by preventing nuclear factor-kappa B (NF-κB) transactivation in cultured chondrocytes [Rosa et al., 2008]. In addition, under some conditions, exogenous NO can stimulate collagen synthesis in cultured human tendon cells [Xia et al., 2006]. *In vivo*, Veihelmann et al. [Veihelmann et al., 2001] demonstrated that NO production by iNOS has anti-inflammatory effects in experimental arthritis, by mediating a reduction of leukocyte adhesion and infiltration. Further research may help to elucidate a potential role for NO-donating agents in OA management.

In the present study, our observations provided the impetus for a novel therapeutic target of the iNOS inhibitor L-NIL, namely, ROS and the LPO product HNE, in OA. In our *in vitro* experiments, NO generation and oxidative stress were induced in human OA chondrocytes treated with IL-1β or SIN (a NO donor). First, we obtained data showing that L-NIL significantly reduced both IL-β-evoked NO and peroxynitrite release as well as iNOS activity in human OA chondrocytes. These findings are in agreement with numerous reports indicating that this drug is a potent inhibitor of NO production via iNOS inactivation in OA *in vitro* and *in vivo*. In cartilage explants, L-NIL markedly curbed chondrocyte apoptosis by obviating NO generation [Pelletier et al., 2001]. In an experimental dog model of OA, oral administration of L-NIL decreased NO production, resulting in marked reductions of major catabolic factors, such as MMPs, IL-1β and peroxynitrite, as well as diminution of cyclooxygenase-2 (COX-2) expression [Pelletier et al., 1999]. Macroscopically, L-NIL diminished cartilage lesion size by approximately 50% on both condyles and plateaus. The histological severity of both cartilage lesions and synovial inflammation was significantly curtailed in L-NIL-treated dogs [Pelletier et al., 1998]. However, these data contrast with those reported by McCartney-Francis et al. [Cartney-Francis et al., 2001] who demonstrated that L-NIL administration exacerbated the chronic inflammatory response, as reflected by profound tissue destruction and bone and cartilage losses. Altogether, such findings warrant caution as to the possible utility of NOS inhibitors in OA therapy, necessitating enhanced knowledge and understanding of the (patho)physiological role of iNOS in OA.

Second, recognizing the important role of LPO products in OA, we tested the hypothesis that inhibition of NO generation in OA chondrocytes culminates in the prevention of HNE generation, a very reactive LPO product. Our findings revealed, for the first time, that L-NIL blocked IL-1β-induced HNE-protein adduct accumulation in isolated chondrocytes and cartilage explants, indicating that NO is involved in LPO. It has been established that NO can promote LPO via peroxynitrite generation. Over the last few

years, reactive LPO products have been at the center of the pathophysiological scene of OA disease. In a previous report, we documented a significant increase of endogenous HNE levels in its adducted form in synovial fluid samples from OA patients compared to control subjects [Morquette et al., 2006]. These data are consistent with the findings of Grigolo and colleagues [Grigolo et al., 2003] and Shah and colleagues [Shah et al., 2005], who observed higher levels of both malondialdehyde and HNE in human articular tissues from OA patients in comparison to healthy subjects. In addition, incubation of isolated OA chondrocytes with tumour necrosis factor-alpha or NO donors (e.g. SIN) provided direct evidence implicating these mediators in HNE production, given that all of them were able to increase HNE/protein adduct levels in cellular extracts [Morquette et al., 2006]. Like NO, HNE has the ability to activate a panoply of factors known for their involvement in OA. For example, in OA cartilage, HNE can provoke transcriptional as well as post-translational modifications of collagen type II (Col II) and MMP-13, resulting in cartilage ECM degradation [Morquette et al., 2006]. In addition, HNE can selectively stimulate COX-2 expression via activating transcription factor/cAMP response element and inhibit iNOS expression via NF-κB inactivation in human chondrocytes [Vaillancourt et al., 2007]. More recently, we demonstrated that HNE-binding to Col II culminates in multiple abnormalities in chondrocyte phenotype and function, indicating its contribution to alteration of cell-ECM interaction in OA [El-Bikai et al., 2010]. Taken together, and in conjunction with the fact that L-NIL abolishes HNE generation and restores redox status, these findings open new avenues for this drug in the treatment of OA patients.

To determine the impact of L-NIL treatment on HNE-metabolizing enzymes in chondrocytes, we conducted additional experiments to verify its effect on GST activity in chondrocytes. As predicted, our data showed that L-NIL prevented IL-1β-induced GST inactivation, suggesting normalization of redox status by its administration. Our results are in agreement with other observations indicating that NO-Derived Oxidants inhibit

GST expression(Wong et al. 2001). The transcriptional suppression of GST by IL-1β is known to be mediated by hepatic nuclear factor [Ng et al., 2007]. Furthermore, our findings strongly suggest that GST inactivation after IL-1β addition could be attributed, in part, to NO generation. The decrease in total GST activity in cartilage can compromise tissue defence against HNE and other electrophiles. Several lines of evidence have revealed that GST is a target of nitrosylation and oxidation by NO and ROS, respectively [Cesareo et al., 2005;Letelier et al., 2010]. While multiple GST isoforms exist with distinct catalytic properties, structural observations have indicated that most cytosolic GST isoforms contain a highly-conserved tyrosine (TYR) residue which is crucial for their catalytic function. It has been shown that thiol group modification at CYS residues affects GST's enzymatic activity. Therefore, NO donor-mediated GST inactivation may possibly arise through interaction with either CYS or TYR residues, or both. It has also been reported that hydrogen peroxide leads to the formation of intra- or inter-subunit disulfide bonds between certain CYS residues within GST amino acid sequences, inactivating this enzyme [Letelier et al., 2006].

For the first time in the literature, our data suggest that NO compromises the detoxification abilities of cartilage and renders it more vulnerable to oxidant injuries through down-regulation of GST activity. GSTA4-4, an isoform of GST, is one of the most important pathways for HNE detoxification by catalyzing the conjugation of this aldehyde with GSH [Awasthi et al., 2004;Balogh and Atkins, 2011]. We have reported recently that gene silencing of GSTA4-4 by small interfering RNA augments the cytotoxic effect of HNE [Vaillancourt et al., 2008]. In contrast, the over-expression of this enzyme in chondrocytes offers significant protection against HNE-induced cell cytotoxicity. Our unpublished data show that GSTA4-4 over-expression in OA chondrocytes blocks different HNE-induced factors known to be involved in the OA process, including MMP-13 and COX-2 expression. Furthermore, we found, in the present study, that L-NIL prevents HNE-induced apoptosis as well as MMP-13 and PGE_2 production. The

molecular mechanism underlying HNE-induced apoptosis as well as PGE_2 and MMP-13 was reported by us previously [Vaillancourt et al., 2008;Vaillancourt et al., 2007;Morquette et al., 2006]. These data can be explained by decreased HNE bioavailability via GST metabolism or perhaps via [HNE/L-NIL] adduct formation. As proposed in Figure 7, we can speculate that HNE trapping by L-NIL occurs via its LYS residue. The α,β double bond of HNE is believed to react spontaneously via Michael-type addition with the sulfhydryl group of CYS, the ε-amino group of LYS, and the imidazole function of HIS, and these adducts are currently considered as the predominant modification of proteins by HNE [Poli et al., 2008].

Furthermore, besides the upstream products of LPO, L-NIL abolished IL-1β-induced ROS generation and p47 NOX phosphorylation in chondrocytes. Two hypotheses could explain these data. The first is based on findings in the literature indicating that NO is important in ROS generation via NOX up-regulation. Compelling but controversial studies have investigated the role of NO in the regulation of ROS production via NOX. Our data are in agreement with the observations of Kaur et al. [Kaur et al., 2004] who reported NOX induction by NO in IL-1β-treated human coronary artery smooth muscle cells. Owayed and colleagues [Owayed et al., 2008] noted an increase in the activity and transcriptional regulation of NOX-1 and gp91phox by NO in peripheral blood lymphocytes from asthmatic patients. However, others studies presented additional evidence that prolonged exposure of human endothelial cells to NO donors causes sustained suppression of superoxide production via inhibition of NOX activity through S-nitrosylation of p47phox [Harrison et al., 2010;Selemidis et al., 2007]. Therefore, the second hypothesis is based on the ability of NOS to generate ROS (Fig. 7). The enzyme can produce large amounts of ROS when deprived of its critical co-factor BH_4 or its substrate L-arginine. Limitation of substrate availability leads to iNOS uncoupling and ultimately to ROS formation [Gielis et al., 2011]. Increased iNOS expression in the presence of limited substrate availability may result in iNOS uncoupling [Otani,

2009;Wells and Holian, 2007], thus potentially promoting ROS formation and, consequently, LPO. It has been reported that iNOS inhibition by N- (3-(aminomethyl)benzyl)acetamidine attenuates superoxide anion formation and oxidative damage [Heusch et al., 2010]. In this state, often referred to as NOS uncoupling, electron flow through the enzyme culminates in reduced molecular oxygen at the prosthetic heme site rather than NO formation [bu-Soud et al., 1994].

In summary, our study demonstrated that L-NIL inhibited NO and ROS generation and, consequently, LPO in OA chondrocytes. Moreover, L-NIL administration was shown to play a role in redox status normalization via the prevention of GST inactivation in these cells. Our findings suggest that L-NIL may represent a new therapeutic approach to chronic autoimmune arthritis. An overview of the different mechanisms mediating HNE production and L-NIL action is proposed (Fig. 7).

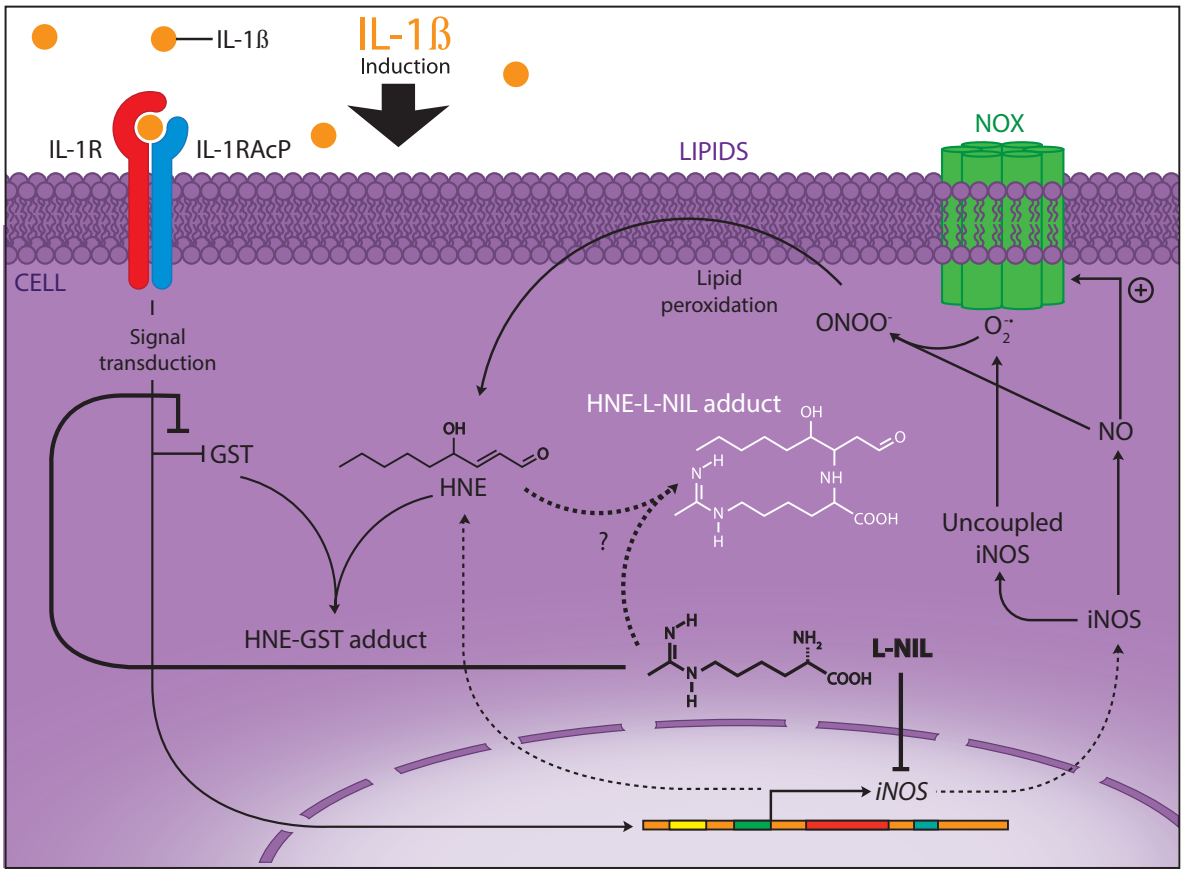

Figure 7: Overview of different mechanisms mediating 4-hydroxynonenal (HNE) production and N-iminoethyl-L-lysine (L-NIL) action. Superoxide anion could be produced by NADPH oxidase (NOX), under NO effect, or by NOS uncoupling. NO mainly interacts with superoxide anion to form peroxynitrite, which further induces lipid peroxidation (LPO). The iNOS inhibitor L-NIL has the ability to abolish HNE levels via (i) NO reduction, (ii) possible [HNE/L-NIL] adduct formation, and (iii) GST up-regulation.

2.6 Acknowledgements

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CHAPTER 3: Discussion

NO as therapeutic target

For a years now, a large number of studies have investigated NO as a therapeutic target in pathologies involving inflammation (Ricciardolo & Sterk 2004; McCartney-Francis & Allen 1993; Pacher et al. 2007; Hansel et al. 2003). NO has been of particular interest in articular pathologies, more specifically OA, due to its heightened levels in the joint fluids of patients with this disease (Abramson 2008). This phenomenon has also been observed in experimental animal OA model (Pelletier et al. 1998). NO overproduction in OA chondrocytes is most likely the result of a snowball effect involving biomechanical stress and redox signals followed by inflammation. Initial stress prompts NO and proinflammatory cytokine production, which in turn activates more NO production, leading to a positive feedback loop. Furthermore, NO has been shown to cause MMP activation, inhibition of proteoglycan and collagen synthesis as well as apoptosis and inflammatory responses in chondrocytes (Abramson 2008). All these make NO an interesting target in OA treatment/prevention. This is why we have focused this study on the reduction of NO production through selective inhibition of its main source, iNOS, by L-NIL.

iNOS inhibition modulates LPO product generation

To imitate knee OA conditions, we started off by inducing NO generation and oxidative stress in human OA chondrocytes by treating them with either IL-1β or SIN (a NO donor). Our results showed a significant increase in NO and HNE/protein adducts with both IL-1β and SIN. We decided to continue investigations with IL-1β treatment since it is naturally present in elevated levels in OA articular cartilage. Afterwards, we needed to make sure that our selective iNOS inhibitor, L-NIL, really did prevent NO production and iNOS activation. The data we obtained concurred with previous reports and confirmed L-NIL's potent ability to suppress both NO release and iNOS activity. Next, we showed that L-NIL can block peroxynitrate generation by observing a decreased in

nitrotyrosine protein adducts. Since $ONOO^-$ it is a key actor in LPO, this indicated that we could also expect a drop in HNE production. Further investigation indeed showed a significant reduction in HNE/protein adduct accumulation in isolated chondrocytes and cartilage explants. This was an interesting find since our laboratory has previously shown HNE's implication in increased inflammation leading to matrix degradation and cell death(Vaillancourt et al. 2008; Morquette et al. 2006). Study into the effects of iNOS inhibition on HNE metabolism showed that addition of L-NIL almost completely abolished IL-1β ability to supress GST, thus making it available for HNE detoxifying. This indicates that NO might play a role in IL-1β induced inactivation GST. In fact, some studies have revealed that GST is a target of nitrosylation and oxidation by NO and ROS, respectively (Cesareo et al. 2005; Letelier et al. 2010).

Inhibition of iNOS blocks pathophysiological effects of HNE

In the present study, we also investigated the consequences of L-NIL addition on three of the most problematic pathophysiological effects of HNE: cell death, MMP-13 and PGE_2 production. We found that OA chondrocyte treatment with L-NIL prevented all three. This did not come as a surprise since we had already established a decrease in HNE/protein adducts. It is probable that an increase in HNE metabolism by now available GST, thus a decrease in HNE bioavailability, is mainly responsible for this protective effect of L-NIL. It is however also possible that HNE might be unavailable due to [HNE/L-NIL] adduct formation. Similar "trapping" phenomenon of HNE by carnosine has already been described by our laboratory (El-Bikai et al. 2010).

L-NIL reduces oxidative stress by preventing ROS generation

Finally, we wondered if L-NIL had an effect on ROS generation because of their implication in both LPO and GST inactivation. ROS levels were found to be abolished by L-NIL. This might be caused by suppression of NOX activation, which has been shown to be induced by NO in IL-1β-treated human coronary artery smooth muscle cells (Kaur et al. 2004). ROS production can also be attributed to iNOS uncoupling through the increase expression of iNOS in OA chondrocytes (Gielis et al. 2011; Otani 2009; Wells & Holian 2007). It is thus also possible that the decrease in ROS observed after L-NIL addition is directly caused by the decrease in iNOS expression.

Though all of our data seem promising, it is important to keep in mind that McCartney-Francis et al. reported that L-NIL administration exacerbates the chronic inflammatory response, as reflected by profound tissue destruction and bone and cartilage losses (McCartney-Francis et al. 2001). Altogether, such findings warrant caution as to the possible utility of NOS inhibitors in OA therapy, necessitating better understanding of the role of iNOS in OA.

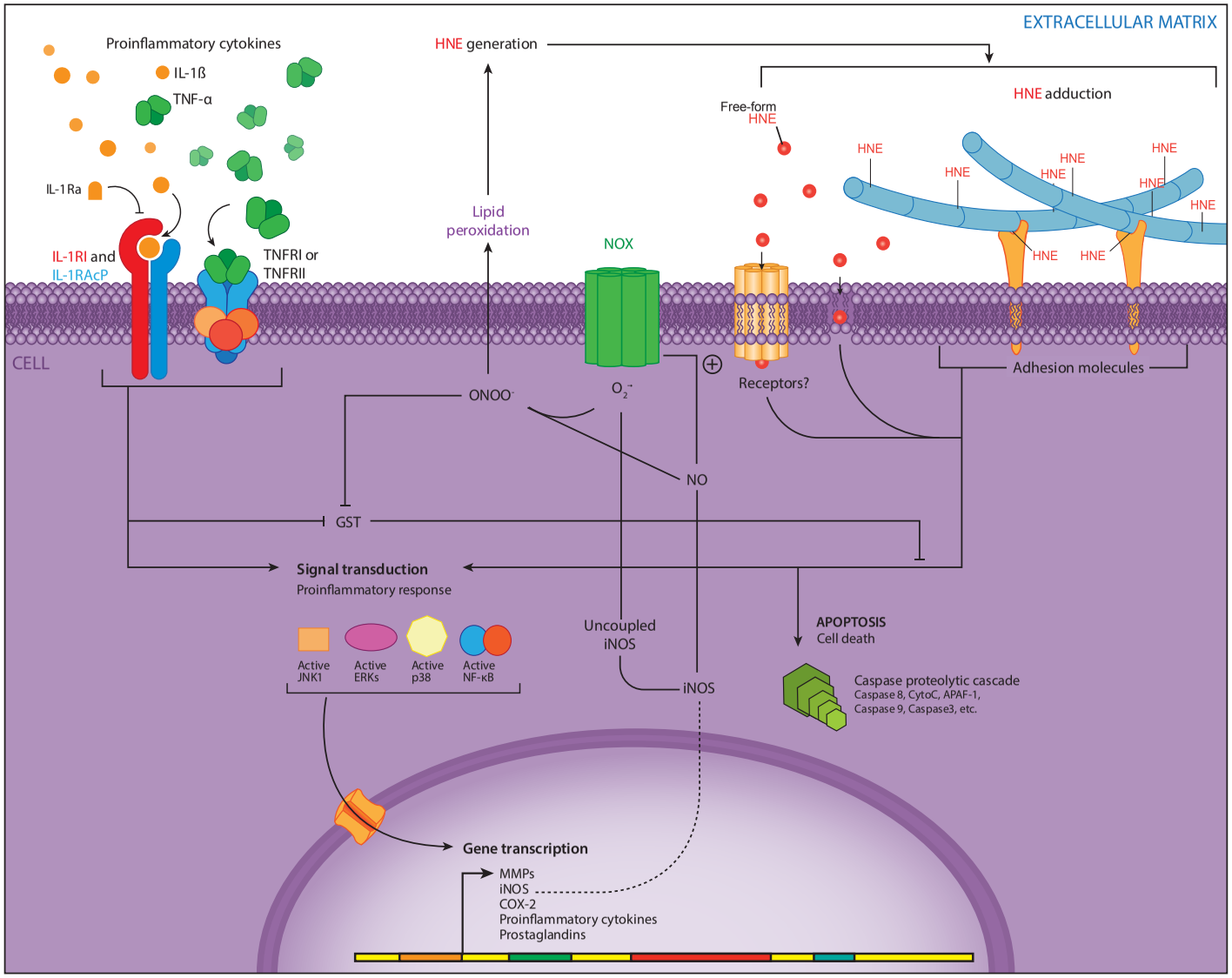

Figure 19. Overview the cellular pathways of studied molecules

Overview of the different cellular pathways explored in this study. .

CHAPTER 4: Conclusion

Because NO plays such a prominent role in OA, we have studied the outcome of selectively inhibiting the enzyme most responsible for its production in OA patients, namely iNOS. To do so, we investigated how L-NIL affected several key aspects of OA pathophysiology. Doing so, we have demonstrated that L-NIL inhibited ROS generation and consequently LPO in OA chondrocytes. Since it prevented LPO, it also abolished HNE production. Moreover, L-NIL administration was shown to play a role in redox-status normalization via preventing GST inactivation in these cells. This probably explains how addition of L-NIL greatly reduced pathological effects of HNE such as cell death as well MMP-13 and PGE_2 upregulation. Another theory is that L-NIL might be trapping HNE by forming an L-NIL/HNE adducts as carnosine does (see Figure 20).

Either way, these findings suggest that L-NIL may represent a new therapeutic approach to the treatment of chronic autoimmune arthritis. Further investigation should be done to better understand L-NIL's mechanism of action and potential side effect *in vivo*. Tough Pelletier and al. have done an OA dog model *in vivo* study with oral administration(Pelletier et al. 1998); it could be interesting to see how local injection might provide a more specific result with a much lower likeliness to have a systemic impact.

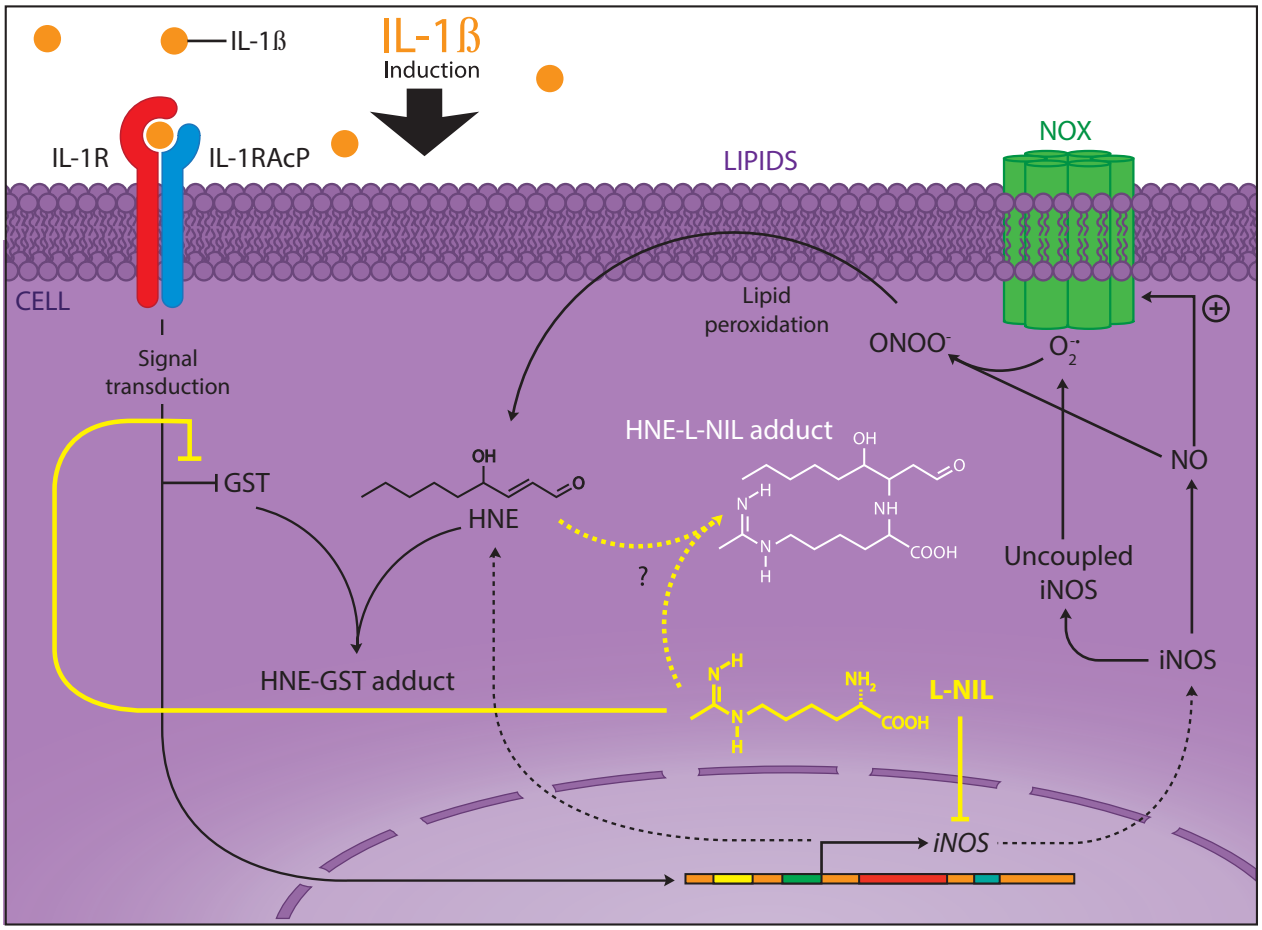

Figure 20. Overview of L-N^6- (l-iminoethyl)lysine effect on 4-hydroxynonenal (HNE)

Overview of different mechanisms mediating 4-hydroxynonenal (HNE) production and N-iminoethyl-L-lysine (L-NIL) action. Superoxide anion could be produced by NADPH oxidase (NOX), under NO effect, or by NOS uncoupling. NO mainly interacts with superoxide anion to form peroxynitrite, which further induces lipid peroxidation (LPO). The iNOS inhibitor L-NIL has the ability to abolish HNE levels via (i) NO reduction, (ii) possible [HNE/L-NIL] adduct formation, and (iii) GST up-regulation (Bentz et al. 2012).

CHAPTER 5: Cited literature

Cited literature

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