Investigation of Chondroprotective Mechanisms of Selenium

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
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ABSTRACT

Selenium (Se) is an essential trace element and metalloid involved in several key metabolic activities: protection against oxidative damage, regulation of immune and thyroid function, and fertility. Several recent lines of evidence from epidemiology, genetic, and transgenic animal studies suggest that Se may play a protective role in Osteoarthritis (OA). However, the exact protective mechanism of Se is still unclear.

In this study, we hypothesized that Se exerts its chondroprotective benefit via an anti-oxidative and anti-inflammatory effect mediated by specific selenoproteins that neutralize cytokine-induced inflammatory responses in chondrocytes. We established an *in vitro* system for studying the effect of Se in the chondrosarcoma cell line SW-1353 and in human primary chondrocytes. Selenomethionine (SeMet) induced gene expression and enzyme activity of both antioxidative enzymes glutathione peroxidase (GPX) and thioredoxin reductase (TR) in SW-1353 cells. Our data suggest that Se may be protective against oxidative stress through regulation of the activity of these antioxidative enzymes.

As IL-1β is one of the primary pro-inflammatory cytokines contributing to the progression in OA, we next investigated the effect of Se on the gene expression induced by physiological doses of IL-1β. SeMet inhibited IL-1β-induced catabolic gene expression of matrix metalloproteinase 1 (MMP1) and MMP13 as well as total MMP activity in chondrocytes. Similarly, SeMet inhibited

chondrocyte gene expression of IL-1β-induced nitric oxide synthase (iNOS) and cyclooxygenase (COX2) with corresponding reductions in nitric oxide (NO) and prostaglandin E2 (PGE_2) production. In addition, SeMet pretreatment attenuated the IL-1β-induced activation of p38 MAPK but not the ERK, JNK or NFkB pathways. Taken together, our results suggest that Se inhibits IL-1β-induced expression of inflammatory and catabolic genes, partly through inhibition of IL-1β cell signaling.

Since Se may function through selenoproteins, we evaluated the role of three specific major selenoproteins, GPX1, TR1 and DIO2, in modifying the inflammatory response stimulated by IL-1β in chondrocytes by RNA interference. Based on RNA interference results, DIO2 and TR1 mediated the inhibitory effect of SeMet on IL-1β-induced COX2 gene expression, while GPX1 did not show a significant inhibitory effect on Se. Depletion of DIO2 increased the IL-1β-induced COX2 gene expression. This suggests that DIO2 may negatively modulate the IL-1β response. Our data also suggest that part of this inhibitory effect of DIO2 could be through direct regulation of IL-1β gene expression. These results highlight a potential new role of DIO2 in modulating the inflammatory response in chondrocytes

In summary, the results of this study suggest that Se may exert its chondroprotective effect through specific selenoproteins which neutralize oxidative stress and modify the inflammatory response in chondrocytes.

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LIST OF ABBREVATIONS

COX2	cyclooxygenase-2
DIO	iodothyronine deiodinase
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ERK	extracellular-signal-regulated kinases
GPX	glutathione peroxidase
HEPES	N-2-Hydroxyethylpiperazine-N'-ethanesulfonic acid
IL-1β	interleukin-1β
IRB	Internal Review Board
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
KBD	Kashin-Beck Disease
LPS	lipopolysaccharide
LXR	liver X receptor
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTT	3-*(*4,5-Dimethylthiazol-2-yl*)*-2,5-diphenyltetrazolium bromide

NADPH	nicotinamide adenine dinucleotide phosphate
NFkB	nuclear factor-kappa B
NMD	nonsense-mediated mRNA decay
NO	nitric oxide
OA	osteoarthritis
OD	optical density
PCR	polymerase chain reaction
PGE_2	prostaglandin E2
RA	rheumatoid arthritis
RNAi	RNA interference
ROS	reactive oxygen species
RT	reverse transcriptase
SBP2	SECIS-binding protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	selenium
Sec	selenocysteine
SECIS	selenocysteine insertion sequence
SeMet	selenomethionine
siRNA	short interfering RNAs
TR	thioredoxin reductase

tRNA	transfer ribonucleic acid
TXNIP	thioredoxin-interacting protein
T4	thyroxine
T3	3,3’,5’-triiodothyronine
TNFα	tumor necrosis factor-alpha

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I would like to thank my advisor, Dr. Virginia B. Kraus, for all of her generous guidance and support during my graduate studies. She has been not only an excellent mentor but also a great friend with whom I could share happiness and worries. Her positive thinking, patience, enthusiasm and encouragement have helped me through many difficulties during my graduate career. In her, I see the perfect balance of a good scientist, mentor, wife and mother at the same time. I hope these good characteristics will be ones that I can develop in my future.

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CHAPTER 1

Introduction

1.1 Historical Impact of Selenium

In the early 19th century, the trace element selenium was observed as a reddish substance in the residue remaining after the manufacture of sulphuric acid. The residue had an unpleasant “rotting radishes” odor and blistered the skin when touched. The substance was initially misclassified as tellurium until 1817 when Swedish chemist, Jons Jakob Berzelius discovered it showed properties distinct from tellurium. Berzelius named it “selenium” after the moon Goddess Selene in Greek mythology (Reilly 2006).

Selenium was originally considered a toxin in the 1930’s when scientists noted that livestock grazing on high selenium soil developed symptoms of staggers disease and alkali disease, which included impaired vision as well as weight and hair loss (Birringer, Pilawa et al. 2002). For this reason, selenium was considered a poison until 1957 when Schwarz and Foltz discovered that selenium prevented liver necrosis in vitamin-deficient rats (Brown and Arthur 2001). Numerous studies subsequently established selenium as an essential element in the human body. In the 1970s, Chinese scientists showed that severe selenium deficiency could contribute to a cardiomyopathy known as Keshan Disease and a disabling chondronecrosis known as Kashin-Beck Disease (1979; Flohe, Andreesen et al. 2000; Birringer, Pilawa et al. 2002) in selenium deficient areas. Symptoms of these diseases improved when the diet was supplemented with selenium. In 1973, the first biologically active form of selenium, glutathione

peroxidase, a key component in the antioxidant defense system, was identified (Flohe, Gunzler et al. 1973).

1.2 Effects of Selenium Deficiency and Toxicity

Selenium has been described as a “two-faced element” (Reilly 2006). In trace amount, it is considered to be an essential nutrient for the human body. In excess, it becomes a toxin. The recommended daily allowance for selenium is 55 μg per day for adults in the US (NIH-OoDS 2010) to prevent deficiency. Selenium deficiency may result in hypothyroidism, heart disease, and a weakened immune system (Combs 2000; Zimmermann and Kohrle 2002). In addition, selenium deficiency may predispose the body to other illnesses when triggered by other nutritional deficiencies, or metabolic or infectious stresses (Beck, Levander et al. 2003). In the northern part of China, severe selenium deficiency is associated with Keshan Disease, which causes heart malfunction, and Kashin-Beck Disease, which causes joint deformations and short stature in selenium deficient regions (Holben and Smith 1999).

In contrast, too much selenium intake can induce a disease status called selenosis. Symptoms of selenosis include diarrhea, fatigue, hair loss, brittle nail, irritability, depression and neurological deterioration (Holben and Smith 1999). The Institute of Medicine of the National Academy of Science recommends a

maximal intake level for selenium at 400 µg per day for adults to prevent the risk of developing toxicity (Institute of Medicine 2000).

1.3 Function of Selenium

Selenium is known to be involved in several key metabolic activities in the human body that are important in preventing certain chronic diseases (Rayman 2000; Reilly 2006). It protects against oxidative stress and regulates production of active thyroid hormone. It has been hypothesized that the antioxidant role of selenium prevents oxidation of lipids and platelet aggregation (Neve 1996). In addition, an increased intake of selenium is associated with reduced risk in developing cardiovascular diseases (Boosalis 2008). Moreover, an adequate selenium uptake was found important for proper function of the immune system and anti-viral defense (Rayman 2000), as well as beneficial in inflammatory conditions such as pancreatitis, rheumatoid arthritis and asthma. Selenium is critical for sperm vitality (Behne, Weiler et al. 1996) and it may prevent miscarriage (Barrington, Taylor et al. 1997). Evidence suggests that selenium has a role in brain function as deficiency results in an altered turnover rate of neurotransmitters, and it may be associated with adverse mood states such as depression, anxiety and Alzheimer's disease (Rayman 2000). Selenium has also been proposed as insulin-mimetic (Stapleton 2000) in diabetes for glucose balance between bloodstream and peripheral tissues. Moreover, a high, but not

toxic, level of selenium intake may reduce the risk of developing cancer (Boosalis 2008).

1.4 Selenium Uptake and Metabolism

Selenium in our diet is mainly absorbed in the lower part of the small intestine. Inorganic forms of selenium include selenate, which is absorbed by means of a sodium-mediated carrier transport mechanism, and selenite, which is absorbed by passive diffusion (Fairweather-Tait 1997). In contrast, absorption of selenomethionine, an organic form of selenium found in food, including cereals, grains, fish and certain vegetables, is active, sharing the same enzyme transport system with methionine (McConnell and Cho 1965). Ingested selenium is transported from the intestine to the liver. It is first reduced to selenide and then either bound to alpha and gamma globulin or it is incorporated into selenoprotein P and transported through the bloodstream to different organs and target tissues (Reilly 2006). Selenium can be incorporated either specifically as selenocysteine into selenoprotein or non-specifically as selenomethionine in the place of methionine. The metabolism of ingested selenium is summarized in Figure 1.1.

Reports suggest that selenomethionine is the most readily absorbed form in that it has a higher retention rate in the human body compared to the inorganic form selenite (Schrauzer 2000). The non specific incorporation process at the place of methionine serves as a vehicle for a selenium reservoir in tissues

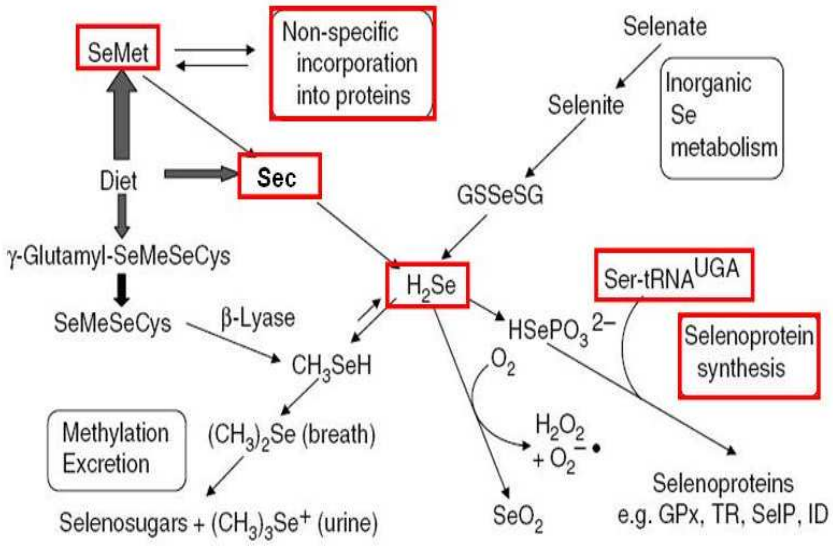


Figure 1.1 The metabolism of dietary forms of selenium.
SeMet, selenomethionine, Sec, Selenocysteine; SeMeCys, selenomethyl-SeCys; GSSeSG, selenodiglutathione; GPX, glutathione peroxidase; TR, thioredoxin reductases; Sel P, selenoprotein P; ID, iodothyronine deiodinases. Red boxes highlight the key steps for non-specific incorporation of selenomethionine into proteins or specific incorporation of selenocysteine into selenoproteins. This figure is modified from Rayman (2005).

(NIH-OoDS 2010) and provides a slow release pool of selenium during protein turnover (Thompson 2001). Selenomethionine can also be converted to selenocysteine (via a trans-sulphuration pathway) followed by metabolism to release inorganic selenium as hydrogen selenide (H_2Se) (Thompson 2001). Selenide can be further converted into selenocysteine for specific incorporation into catalytic sites of selenoproteins through a highly-regulated process described in the following section.

1.5 Selenium Incorporation and Selenoprotein Synthesis

Selenium, incorporated in the form of the 21st amino acid selenocysteine, functions at key catalytic sites of selenoproteins. This incorporation of selenium into selenoproteins can be divided into two main steps: 1) synthesis of selenocysteine on the specific selenocysteine tRNA $^{[Ser]\ Sec}$ for subsequent incorporation into selenoprotein and; 2) co-translational incorporation of selenocysteine into selenoproteins.

In contrast to other amino acids, selenocysteine is synthesized on its own tRNA, designated as Sec tRNA $^{[Ser]\ Sec}$, through modification of serine. Intracellular selenide is first activated by selenophosphate synthetase 2 to form monoselenophosphate. Phosphoseryl tRNA kinase phosphorylates the Seryl-tRNA $^{[Ser]\ Sec}$ complex, which then reacts with monoselenophosphate to yield

selenocysteine by selenocysteine tRNA synthase. A schematic illustration of Sec biosynthesis is presented in Figure 1.2 (Papp, Lu et al. 2007).

Selenium is incorporated into selenoproteins through a remarkable machinery in which the UGA stop code is recognized as a sense codon for inserting selenocysteine. This recoding system requires a specific RNA secondary structure called selenocysteine insertion sequence (SECIS) element in the 3' untranslated regions of selenoprotein messages. These SECIS elements recruit the SECIS-binding protein 2, SBP2, as a scaffold to form a complex with a selenocysteine specific elongation factor and its own tRNA, Sec tRNA $^{[Ser]\ Sec}$ Figure1.3 (Berry 2005).This specific complex facilitates a cotranslational incorporation of selenocysteine into a growing polypeptide chain of selenoproteins.

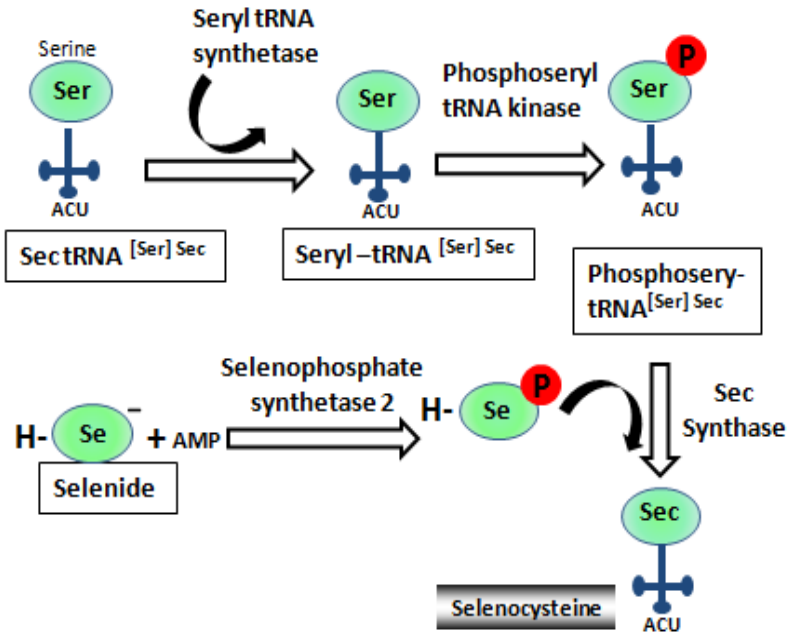


Figure 1.2 Selenocysteine biosynthesis pathway in mammalian cells. Modified from Papp, Lu et al. (2007).

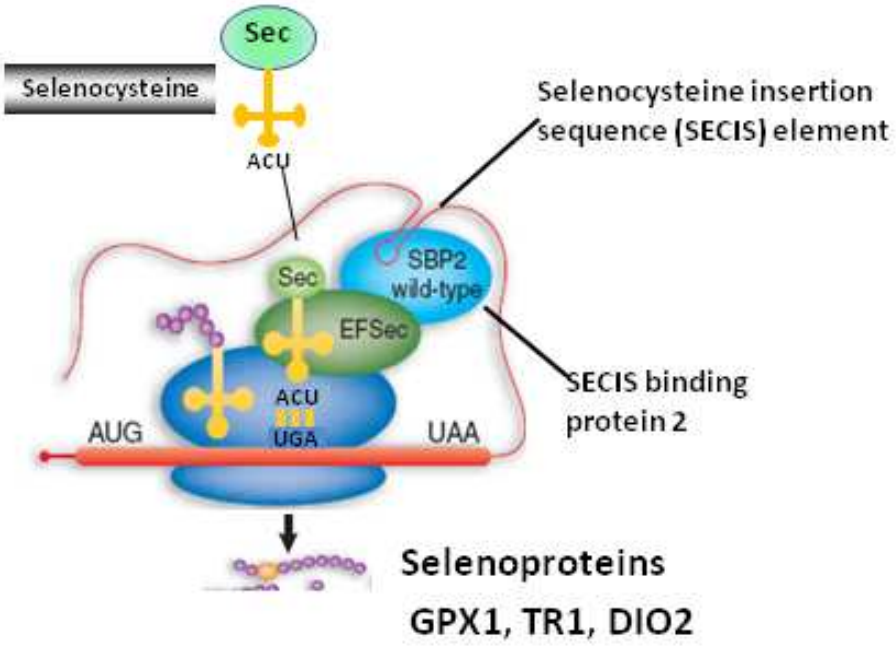
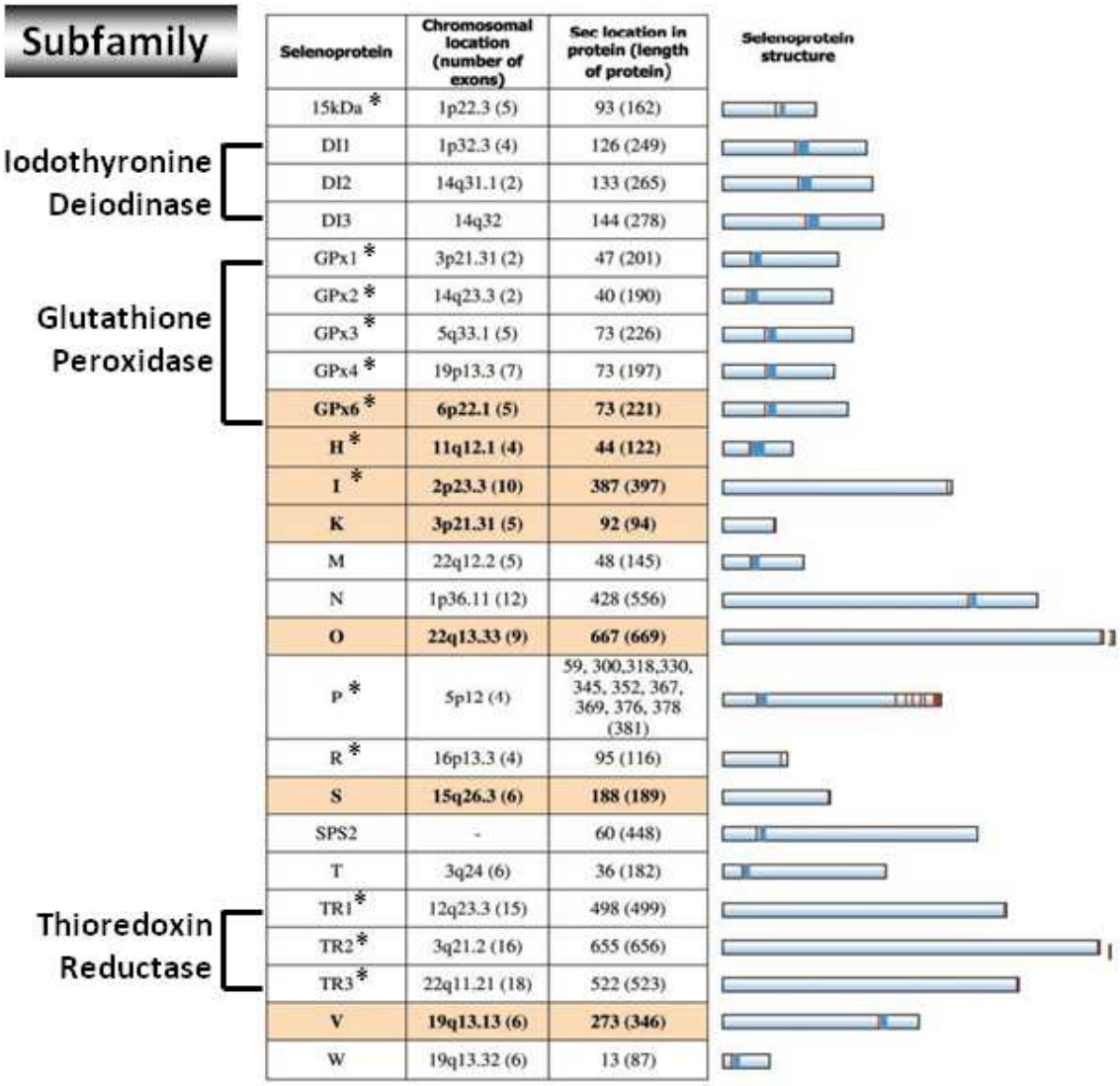


Figure 1.3 Selenoprotein synthesis. The SECIS element in the 3'untranslated region of the mRNA (hairpin stem loop) recruits SBP2 (blue), which then recruits EFSec (green) and specific tRNA (yellow). The complex interacts with ribosome to recode UGA as selenocysteine. Modified from Berry (2005).

1.6 Selenoproteins

To date, 25 selenoproteins have been identified in human, and 24 in rodents (Figure 1.4) (Kryukov, Castellano et al. 2003) (Reeves and Hoffmann 2009). Expression levels and activities of selenoproteins are highly related to selenium availability in the human body (Hara, Shoji et al. 2001; Papp, Lu et al. 2007). Glutathione peroxidase and thioredoxin reductase are two major subfamilies of selenoproteins. Other members of selenoproteins include selenoprotein H, selenoprotein M, selenoprotein O, selenoprotein P, selenoprotein R and selenoprotein W. Selenoprotein P is unique among other members as it is the only one containing multiple selenocysteines. Selenoprotein P has been demonstrated to have a role in selenium transport in plasma and tissues. All of the above selenoproteins, accounting for more than half of all selenoproteins, have been shown to have antioxidant activity (Kryukov, Castellano et al. 2003; Reeves and Hoffmann 2009). The iodothyronine deiodinases, another major subfamily of selenoproteins, regulate production of bioactive thyroid hormones (Reeves and Hoffmann 2009).



Selenoprotein	Chromosomal location (number of exons)	Sec location in protein (length of protein)
15kDa *	1p22.3 (5)	93 (162)
DI1	1p32.3 (4)	126 (249)
DI2	14q31.1 (2)	133 (265)
DI3	14q32	144 (278)
GPx1 *	3p21.31 (2)	47 (201)
GPx2 *	14q23.3 (2)	40 (190)
GPx3 *	5q33.1 (5)	73 (226)
GPx4 *	19p13.3 (7)	73 (197)
GPx6 *	**6p22.1 (5)**	**73 (221)**
H *	**11q12.1 (4)**	**44 (122)**
I *	**2p23.3 (10)**	**387 (397)**
K	**3p21.31 (5)**	**92 (94)**
M	22q12.2 (5)	48 (145)
N	1p36.11 (12)	428 (556)
O	**22q13.33 (9)**	**667 (669)**
P *	5p12 (4)	59, 300,318,330, 345, 352, 367, 369, 376, 378 (381)
R *	16p13.3 (4)	95 (116)
S	**15q26.3 (6)**	**188 (189)**
SPS2	-	60 (448)
T	3q24 (6)	36 (182)
TR1 *	12q23.3 (15)	498 (499)
TR2 *	3q21.2 (16)	655 (656)
TR3 *	22q11.21 (18)	522 (523)
V	**19q13.13 (6)**	**273 (346)**
W	19q13.32 (6)	13 (87)

Figure 1.4 Summary of human selenoprotein genes. Proteins are shown in alphabetical order and proteins with antioxidant activity are marked with an asterisk. On the right, relative lengths of proteins are shown and selenocysteine locations within the proteins are indicated by red vertical lines. Modified from Kryukov, Castellano et al. (2003)

Glutathione Peroxidase (GPX)

The biochemical function of GPXs is to reduce hydrogen peroxide and lipid peroxide into water and their corresponding alcohol, respectively (Lei, Cheng et al. 2007). GPX1, or cellular GPX, was the first identified selenoprotein and is the most abundant selenoperoxidase, being expressed in almost all tissues (Lei, Cheng et al. 2007). GPX3 is expressed in various tissues as a secreted glycoprotein in extra-cellular fluids (Takahashi, Avissar et al. 1987) and it constitutes approximately 20% of selenium in the plasma (Koyama, Omura et al. 1999). GPX4 is unique among other members, as it is the only GPX that reduces phospholipids and hydroperoxides and has a structural role essential for sperm vitality after sperm maturation (Savaskan, Ufer et al. 2007). Alternate expression of GPXs have been shown in osteoarthritic cartilage (Aigner, Fundel et al. 2006): GPX1 and GPX4 are downregulated approximately 1.2 fold in cartilage lesions with moderate to severe late-stage osteoarthritis compared with normal samples. These findings highlight the potential protective role of selenium and its selenium-containing GPX in osteoarthritis progression.

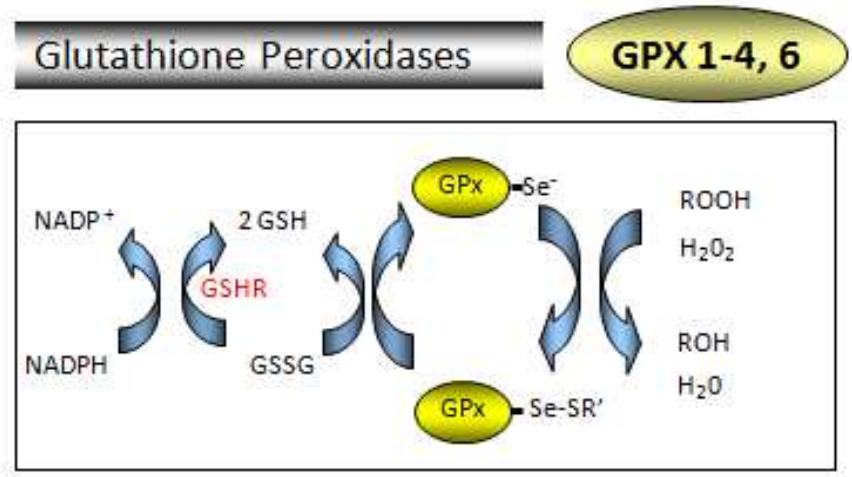


Figure 1.5 Electron coupling of glutathione reductase and GSH with mammalian glutathione peroxidases. GPXs couple with glutathione reductase (GSHR) and catalyze the reduction of organic hydroperoxides and hydrogen peroxide, protecting cells against oxidative damage. Modified from Papp, Lu et al. (2007)

Thioredoxin Reductase (TR)

TR enzymes are members of the pyridine nucleotide disulfide oxidoreductase family which maintain the reduced state of thioredoxin. C-terminal selenocyteine residues at the enzymatic active site of TRs catalyze the reduction of oxidized thioredoxin in an NADPH-dependent manner (Figure 1.6). Thioredoxin is a key factor in cellular redox regulation by serving as a cofactor in disulfide bond reduction. The reduced state of cysteine groups of various proteins is essential for DNA synthesis, oxidative defense and protein folding (Papp, Lu et al. 2007). Cellular TR1 and mitochondrial TR2 are both housekeeping reductases and ubiquitously expressed in tissues. TR3 shows specific expression in testis (Behne and Kyriakopoulos 2001). Thioredoxin-interacting protein (TXNIP) has been shown to be downregulated in osteoarthritic cartilage suggesting the potential protective role of thioredoxin and thioredoxin reductase in maintaining normal cartilage (Aigner, Fundel et al. 2006).

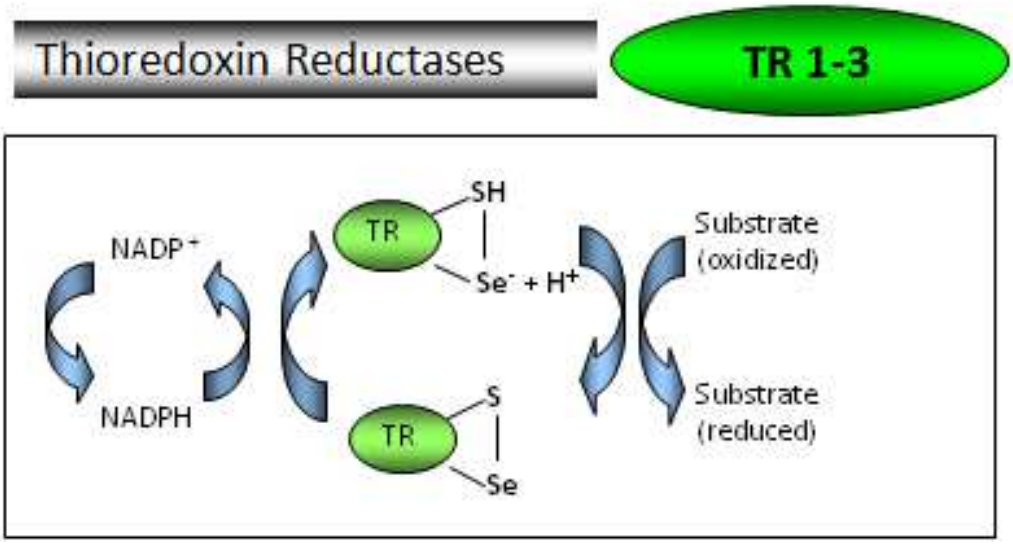


Figure 1.6 Electron coupling of NADPH with mammalian thioredoxin reductase. Mammalian TRs target a wide range of substrates including, macromolecules in which disulfide bonds play a critical functional role and low molecular weight compound like thioredoxin. Modified from Papp, Lu et al. (2007)

Iodothyronine Deiodinase (DIO)

Three members of the iodothyronine deiodinase (DIOs) family are essential for regulating active thyroid hormone level. DIO1 and DIO3 are plasma membrane-bound, whereas DIO2 is located in the endoplasmic reticulum membrane (Behne and Kyriakopoulos 2001; Papp, Lu et al. 2007). DIO1 and DIO2 are responsible for conversion of the prohormone, thyroxine (T4), to its active form, 3,3',5'-triiodothyronine (T3) through deiodination. DIO3 inactivates T4 and T3 through deiodination, while DIO1 also catalyzes deiodination of T3 to inactive 3,3'-diiodothyronine (T2) (Figure 1.7).

DIO2 is believed to have a major role for local production of T3 in organs or tissues including the brain, brown adipose tissue, pituitary and other peripheral tissues that express this enzyme (Reeves and Hoffmann 2009). DIO2 knockout mice show deficits in thermogenesis (de Jesus, Carvalho et al. 2001), auditory function (Ng, Goodyear et al. 2004) and changes in T3 levels in the brain (Galton, Wood et al. 2007).

A recent genetic study showed a variant of DIO2 is associated with increased risk for developing osteoarthritis (Meulenbelt, Min et al. 2008). DIO3 is suggested, in meta-analyses of genes modulating intracellular T3 levels, to have a possible role in osteoarthritis susceptibility in a related study (Meulenbelt, Bos et al. 2010). These findings suggest that the local availability of thyroid hormone may have a role in pathogenesis of osteoarthritis.

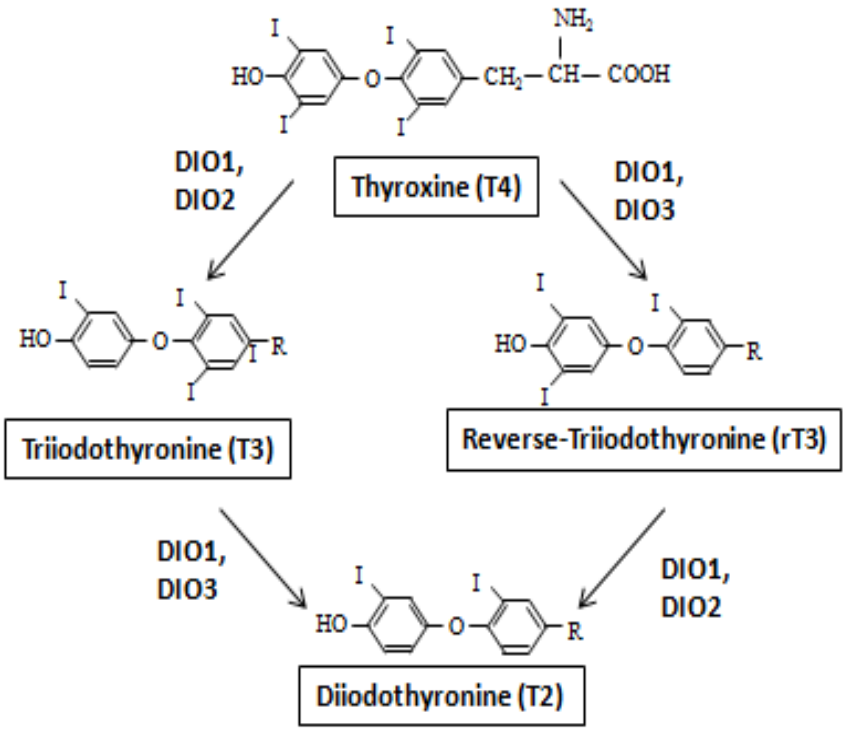


Figure 1.7 Metabolism of thyroid hormone thyroxine by deiodinases. DIO1 and DIO2 catalyze the reduction of T4 to yield the active hormone T3, or reverse T3 to yield T2. DIO1 and DIO3 inactivate by deiodination of T3 to T2. Modified from Papp, Lu et al. (2007).

1.7 Overview of Osteoarthritis

Osteoarthritis (OA) is the most prevalent form of arthritis and results in physical disability for millions of people worldwide (Brooks 2002). In the United States, 21 million adults were affected in 1995, increasing by almost 30% to 27 million in 2005 (Lawrence, Helmick et al. 1998). There is currently no effective therapy against osteoarthritis progression except relieving the symptoms of the disease. Osteoarthritis is highlighted as one of the major problems in the Bone and Joint Decade (2000-2010), which aims to improve the lives of people with musculoskeletal disorders through better prevention and treatment (Harris 2001; Brooks 2002).

Ageing has been considered a major risk factor contributing to osteoarthritis progression (Loeser 2004; Carrington 2005). The disease affects about 60% of men and 70% of women above 65 years of age (Goldring 2006). The incidence and prevalence of the disease increases two to ten-fold from age 30 to 65. (Felson, Lawrence et al. 2000) However, the lack of osteoarthritis in many elderly people suggests that aging is not the sole factor for osteoarthritis. Other risk factors associated with developing osteoarthritis including: systemic factors such as genetics, nutritional deficiency, gender, and bone density; local biomechanical factors such as obesity, muscle disorder, previous joint trauma, and continuous overuse of a joint (Felson, Lawrence et al. 2000; Goldring and Goldring 2007). The common clinical symptoms experienced by OA patients

included: joint pain, stiffness, tenderness, swelling, crepitus (creaking of joints), instability and deformity of the affected joints in late stages (Hochberg 1997) (Hunter and Felson 2006).

1.8 Pathogenesis of Osteoarthritis

Osteoarthritis is considered to be a chronic disease of the joint involving cartilage, synovium, ligaments, bone, meniscus, tendon, and peri-articular muscle (Figure 1.8) (Hunter and Felson 2006). Cartilage destruction is one of the common characteristics of osteoarthritis progression and results in malfunction of the affected joint (Figure 1.9). Normal articular cartilage is comprised of large amounts of extracellular matrix (mainly type II collagen), which is made and maintained by chondrocytes, the sole cell type in the cartilage. During disease progression, net loss of cartilage matrix results from an imbalance between cartilage matrix degradation and synthesis by chondrocytes in the cartilage (Fukui, Purple et al. 2001; Krasnokutsky, Attur et al. 2008). Although many different factors can initiate the development of osteoarthritis, the pathogenesis of osteoarthritis follows a typical pattern. The earliest pathological changes include increased chondrocyte proliferation and synthetic activity. This is followed by increased expression and activation of proteolytic enzymes and matrix proteins in an attempt to repair defects in the cartilage.

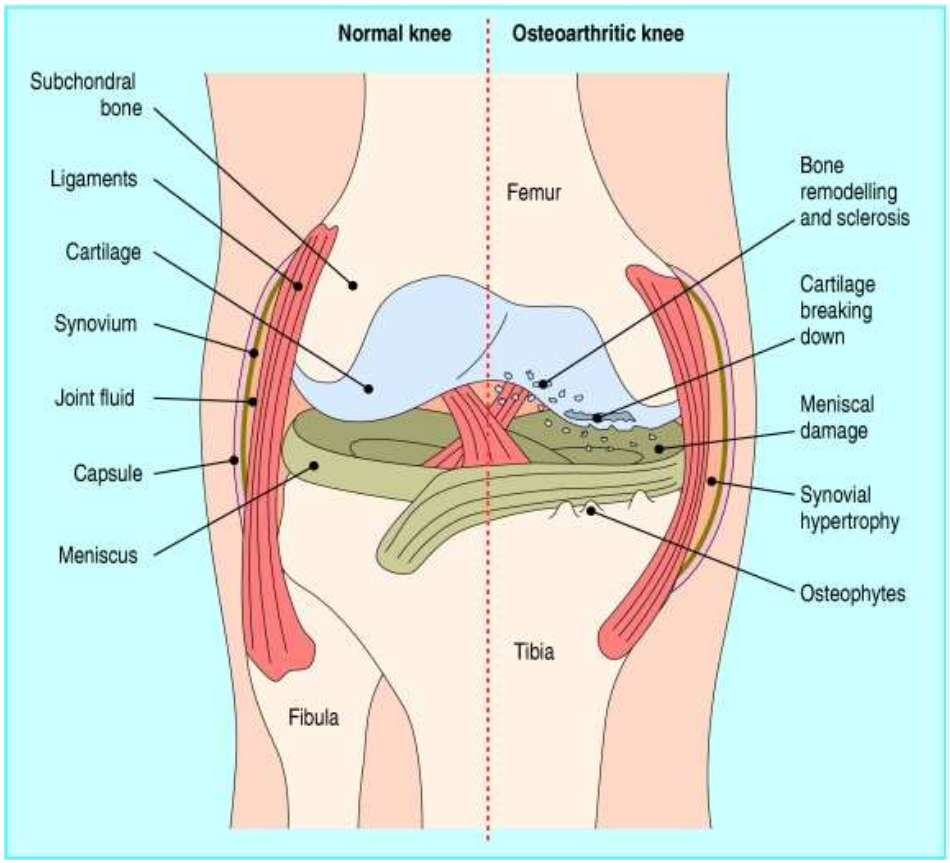


Figure 1.8 Normal structure of the knee (Left) and pathogenic changes observed in OA joints (Right). Adapted from Hunter and Felson (2006).

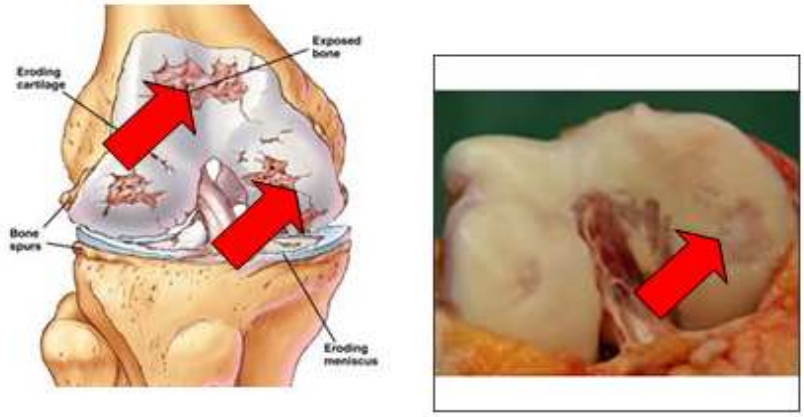


Figure 1.9 Lesion resulting from cartilage destruction during osteoarthritis progression. Adapted from www.vangsnessmd.com/orthoproc_cartilage.htm

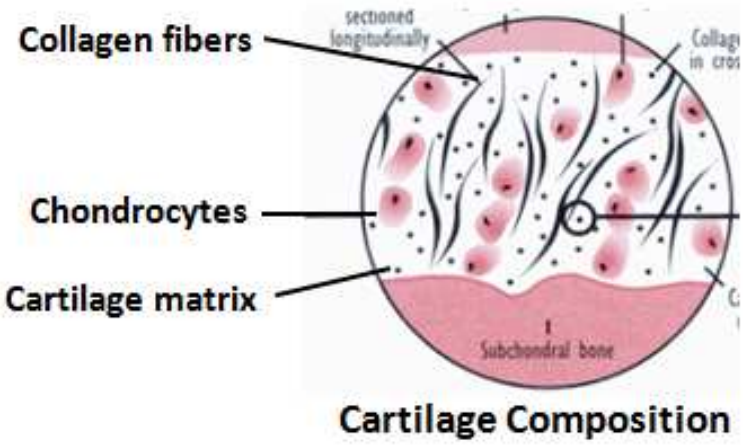


Figure 1.10 Cartilage composition. Normal articular cartilage is composed of chondrocytes, collagen fibers (mainly type II collagen) and cartilage matrix (proteoglycan). Chondrocytes are the only cell type in the cartilage and are responsible for the maintenance of cartilage integrity. Modified from http://www.vetpro.co.nz.

Proteoglycan loss and type II collagen degradation gradually develop in the surface region of articular cartilage and lead to fibrillation. Finally, osteophyte formation and subchondral bone sclerosis, resulting from abnormal anabolic activity, are found at later stages of the disease (Kraus 1997; Sandell and Aigner 2001; Li, Xu et al. 2007).

1.9 Role of Interleukin 1 Beta in the Pathogenesis of Osteoarthritis

Proinflammatory cytokines, such as Interleukin 1 (IL-1) and tumor necrosis factor alpha (TNFα), released from chondrocytes and synovial cells of the joint, have long been considered to be key players in the pathogenesis of OA. Particularly, interleukin 1 beta (IL-1β), has been shown to be upregulated in cartilage and synovial fluid of OA patients (Schlaak, Pfers et al. 1996; Saha, Moldovan et al. 1999). IL-1β deficiency showed protection against OA in a surgically-induced OA mouse model (Goldring and Marcu 2009). Importantly, IL-1β stimulates chondrocytes to produce degradative enzymes, matrix metalloproteinase (MMPs), as well as several key proinflammatory mediators such as prostaglandin E (PGE_2) and nitric oxide (NO) that have been implicated in OA progression (Goldring and Berenbaum 2004; Jacques, Gosset et al. 2006; Daheshia and Yao 2008).

Matrix Metalloproteinase (MMP) and Cartilage Degradation

IL-1β upregulates MMP1, MMP3 and MMP13 in human (Tetlow, Adlam et al. 2001; Fan, Bau et al. 2005), rabbit, bovine and equine chondrocytes (Daheshia and Yao 2008). MMPs are synthesized as inactive proenzymes that require activation for the catalytic domain to achieve full activity (Malemud, Islam et al. 2003). MMP1 and MMP13, classified as collagenases, target native collagen fibers as their substrate. MMP1 cleaves helical region of fibrillar collagens, and shows reactivity with type I, II and III collagens (Goupille, Jayson et al. 1998) (Shingleton, Ellis et al. 2000). MMP13, also known as collagenase 3, cleaves type II collagen more efficiently than other collagens (Knauper, Lopez-Otin et al. 1996; Reboul, Pelletier et al. 1996). MMP13 is considered to play a prominent role in cartilage degradation during OA progression due to its specific potency to degrade the enriched type II collagen network in cartilage. A recent study demonstrated that MMP13 deficiency inhibited cartilage damage in a mouse model of OA (Little, Barai et al. 2009). MMP3, also known as stromelysin, digests type IX collage and proteoglycans (Jacques, Gosset et al. 2006) and it has been shown to be expressed constitutively in human chondrocytes isolated from OA cartilage (Ganu, Hu et al. 1994).

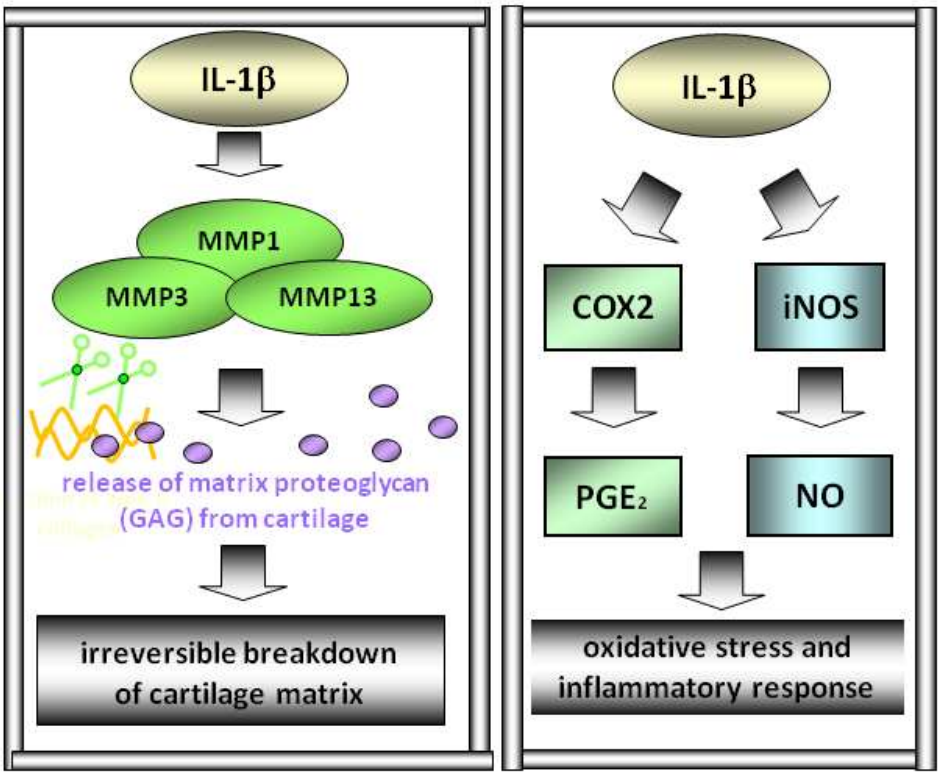


Figure 1.11 Catabolic and inflammatory effects of IL-1β during OA progression. IL-1β induces production of MMPs in chondrocytes and synovial cells which in turn breaks down the cartilage matrix, resulting in cartilage degradation. IL-1β also stimulates production of key proinflammatory mediators such as PGE_2 and NO, which generally cause oxidative stress and catabolic effect in the cartilage, contributing to the further development of OA.

Prostaglandin E2 (PGE_2) in the Development of Osteoarthritis

IL-1β can induce PGE_2 production by stimulating expression of cyclooxygenase-2 (COX2), prostaglandin E synthase 1 (PGES) and phospholipase A2 (Thomas, Berenbaum et al. 2000) (Massaad, Paradon et al. 2000; Masuko-Hongo, Berenbaum et al. 2004). PGE_2 has protean manifestations - some catabolic and some anabolic. This is thought to be mediated by different E-prostaglandin (EP) receptors such as EP_2 and EP_4 (Attur, Al-Mussawir et al. 2008; Li, Ellman et al. 2009). PGE_2 promotes production of MMPs in articular chondrocytes (Bunning and Russell 1989; Tung, Arnold et al. 2002) and synovial fibroblasts (Mehindate, al-Daccak et al. 1995), which in turn causes cartilage degradation. Furthermore, PGE_2 has been shown to modulate osteoclast formation and bone resorption *in vitro* (Lader and Flanagan 1998). EP_4 receptor-deficient mice showed a reduction of cartilage degradation in collagen-induced arthritis (McCoy, Wicks et al. 2002). Besides degrading cartilage, PGE_2 has also been shown to positively modulate type II collagen gene expression in cultured chondrocytes (Goldring, Suen et al. 1996). Previous studies also demonstrated that PGE_2 increased DNA and aggrecan synthesis in a rat chondrocyte cell line (Lowe, Fu et al. 1996). Furthermore, PGE_2 promotes differentiation and proliferation of growth plate chondrocytes (Schwartz, Gilley et al. 1998; Brochhausen, Neuland et al. 2006). Sensitization of peripheral nociceptors by

PGE_2 is believed to induce pain associated with inflammation (Dray and Read 2007).

Nitric oxide (NO) in the Development of Osteoarthritis

IL-1β increases concentrations of NO, another key proinflammatory mediator, via upregulation of inducible nitric oxide synthase (iNOS) in chondrocytes. *In vivo* inhibition of iNOS reduced disease progression in an experimental OA dog model (Pelletier, Jovanovic et al. 1998). NO has been demonstrated to have an impact on cartilage matrix synthesis and degradation in developing OA (Abramson, Attur et al. 2001). NO inhibits collagen and proteoglycan synthesis of cartilage *in vitro* (Taskiran, Stefanovic-Racic et al. 1994), and also induces matrix metalloproteinase synthesis in articular chondrocytes (Sasaki, Hattori et al. 1998). In addition, NO has been shown to reduce IL-1 receptor antagonist (IL-1ra) synthesis by chondrocytes (Pelletier, Mineau et al. 1996). An experimental osteoarthritis model demonstrated that NO is involved in cartilage degradation partially through up-regulation of catabolic factors, interleukin-1-converting enzymes and interleukin-18 synthesis (Boileau, Martel-Pelletier et al. 2002). Incubation of human articular chondrocytes with the NO donor, sodium nitroprusside (SNP), activates apoptotic gene expression (Maneiro, Lopez-Armada et al. 2005). NO can trigger apoptosis by a mitochondria-dependent mechanism that could be mediated through regulation

of expression of apoptotic-related genes such as caspase 3 and 7 (Maneiro, Lopez-Armada et al. 2005; Wu, Chen et al. 2007). Evidence also suggests that NO may increase susceptibility to injury in chondrocytes in response to oxidants (Clancy, Abramson et al. 1997). Another study demonstrated that NO inhibited the chondrocyte response to anabolic growth factor insulin-like factor 1 (IGF-1) (Studer, Levicoff et al. 2000; Loeser, Carlson et al. 2002). Furthermore, 3-nitrotyrosine, a stable product formed by reaction of NO and reactive oxygen species, correlated with IL-1β-induced oxidative stress in aging and osteoarthritic cartilage tissue (Loeser, Carlson et al. 2002).

1.10 Relevance of Selenium in Joint Disease

Selenium and Kashin-Beck Disease (KBD)

Endemic diseases related to Se deficiency in humans include Kashin-Beck Disease (KBD), a severe form of osteoarthropathy endemic to a region extending from northeastern China to Tibet in the southwest, neighboring areas of Russia, and some regions of Vietnam and Korea (Yang, Bodo et al. 1991) affecting as many as 1-3 million people across China (Allander 1994). Pathogenesis of KBD involves necrosis and remodeling of cartilage including growth plates (Kraus). KBD affects multiple joints, including knee, ankle, wrist and elbow. Severely affected cases can cause short stature and disproportionate shortening of the extremities of the patients (Sokoloff 1985). Selenium deficiency

has been considered to be a key predisposing factor for susceptibility to the disease when exposed to environmental stress such as contamination of organic acid and mycotoxin in the diet (Reilly 2006). A variant of GPX1, antioxidative selenoprotein, is found to be associated with susceptibility to KBD in a genetic study (Xiong, Mo et al. 2010). Furthermore, a recent study showed that conditional knockout of the selenocysteine tRNA gene, which is required for selenoprotein synthesis, resulted in skeletal abnormalities and severe chondronecrosis of articular cartilage resembling Kashin-Beck Disease (Downey, Horton et al. 2009). This study highlights the critical role of selenoproteins in skeletal development and the formation of healthy cartilage.

Selenium and Osteoarthritis

Evidence for a role for selenium in OA came from a large population-based study in the United States in which a low but non-deficiency level of selenium was shown to be associated with OA presence and severity (Jordan, Fang et al. 2005; Jordan, Fang et al. 2007). Moreover, a recent genetic study showed that a variant of Deiodinase 2 (DIO2), which encodes a selenoprotein involved in thyroid hormone activation, is associated with the risk of developing OA (Meulenbelt, Min et al. 2008). DIO3, another selenoprotein responsible for inactivation of thyroid hormone, has been suggested to play a potential role in osteoarthritis susceptibility in a meta analysis of genes modulating intracellular

T3 levels (Meulenbelt, Bos et al. 2010). These data highlight that abnormal thyroid hormone levels may contribute to the development of OA. Finally, expression of selenoprotein genes has been shown to be altered in OA cartilage , namely, GPX1 and GPX4, are downregulated approximately 1.2 fold in the cartilage lesions in moderate to severe late-stage OA compared with normal samples (Aigner, Fundel et al. 2006). These findings suggest the potential protective role of selenoproteins in OA progression.

1.11 Potential Protective Role of Selenium in Osteoarthritis

An imbalance in the production of reactive oxygen species in localized compartments has been postulated to play a role in the pathogenesis of a number of degenerative clinical disorders (Ho, Magnenat et al. 1998) including OA. In particular, reactive oxygen species have been shown to induce chondrocyte apoptosis, matrix degradation and other catabolic processes in the cartilage and contribute to the development of OA (Krasnokutsky, Attur et al. 2008). Selenium plays an important role in the host antioxidant defense system through selenoproteins, such as GPX1 and TR1, to attenuate and neutralize reactive oxygen and nitrogen species. This activity is anti-inflammatory and potentially protective against OA. An anti-inflammatory effect of selenium is supported by the roles played by the selenoproteins, GPX and TRs in interfering

eicosanoid metabolism and modulating inflammation (Reilly 2006). Specifically, through the selenium-dependent antioxidant enzymes GPX1 and TRs (Kohrle, Jakob et al. 2005), selenium is expected to inhibit tissue damage due to NADPH oxidase-derived H_2O_2, thereby reducing activation of leukotrienes and possibly counteracting oxidative responses of pro-inflammatory cytokines (Birringer, Pilawa et al. 2002).

There is a possibility that the dysregulation of thyroid hormone expression by selenium deficiency may be a contributing causal pathway for OA. It is difficult to distinguish between the radiological features of hypothyroidism and Kashin-Beck Disease (Moreno-Reyes, Suetens et al. 1998). Hypothyroidism can be associated with arthritis (Liote and Orcel 2000). Because of the fact that selenium is a key cofactor for thyroid hormone (T3) production, the pathogenesis of OA due to Se deficiency may be in part a consequence of inadequate local T3 for the maintenance of chondrocyte health and differentiation states of the calcified cartilage layer. Furthermore, thyroid hormone has direct effects on chondrocytes of the growth plate and may have indirect effects mediated by growth hormone and insulin-like growth factor on the growth plate (Nilsson, Marino et al. 2005). Local conversion of T4 to T3 by DIO2 (dependent on selenium availability) by chondrocytes in the growth plate may contribute to those local effects (Nilsson, Marino et al. 2005). The availability of local thyroid hormone, possibly through

DIO2 and DIO3, has been suggested to play a role in the pathogenesis of symptomatic OA (Meulenbelt, Bos et al. 2010).

1.12 Thesis Summary

In this thesis, we hypothesize that selenium exerts its chondroprotective benefit via an anti-oxidative and anti-inflammatory effect, mediated by specific selenoproteins that neutralize cytokine-induced inflammatory responses. In order to test this hypothesis, our aims were three-fold. Our first aim was to establish a model system for evaluating the effect of selenium in human chondrocytes. Our second aim was to evaluate the effect of selenium on inflammatory gene in human chondrocytes. Finally, our third aim was to evaluate the role of specific selenoproteins as mediator of the anti-inflammatory effect of selenium in human chondrocytes.

The goal of the study in Chapter 2 was to establish a working model for evaluating the effect of selenium on chondrocytes. First, cytotoxicity of two forms of selenium: selenomethionine (SeMet), organic form; and selenite, inorganic form, were examined in SW-1535 chondrosarcoma cells to determine the less toxic form and optimal dose of selenium for our study. The selected form (SeMet) and dose (0.5 µM) of selenium was further tested for toxicity in human primary chondrocytes. We determined the time required for the cell to use up cellular selenium storage obtained from the serum that we use for cell culture, by

measuring selenium protein marker levels and monitoring cell viability. Importantly, we determined the feasibility of SeMet to induce antioxidative enzymes, GPXs and TRs expression and activities, in SW-1353 cells. Finally, we determined the optimal dose range of IL-1β to be used in this study, by measuring the gene expression of MMPs. We also studied the effect of SeMet on IL-1β-induced MMPs expression and activities.

In chapter 3, we chose to investigate potential mechanisms of selenium mediated effects using physiological doses of IL-1β. We examined the effect of selenium in neutralizing the inflammatory effects of IL-1β on NO and PGE_2 production as well as iNOS and COX2 gene expression. Under these conditions we also studied the potential signaling pathways involved in primary human chondrocytes.

In chapter 4, we evaluated the role of specific selenoproteins in regulating inflammatory responses in human chondrocytes. We examined the effect of depleting selenoproteins, GPX1, TR1 and DIO2 on the inflammatory responses in the conditions we established in chapter 3. These included the utilization of RNA interference (RNAi), to determine the role of selenoproteins in mediating the IL-1β-induced inflammatory response through measuring COX2 and IL-1β transcripts levels.

In Chapter 5, we provide a summary of our research findings, conclusions that can be drawn from these results and future directions. Based on our data, we further propose models for chondroprotective mechanism of selenium.

CHAPTER 2

Establishment of a Model System for Evaluating the Effect of Selenium on Human Chondrocytes

2.1 Introduction

Osteoarthritis (OA) is the most common form of arthritis causing disability in the elderly (Brooks 2002; Goldring 2006). Currently, there are no effective therapeutics for OA, and treatment is aimed solely at relieving symptoms. Overproduction of reactive oxygen species (ROS) in localized compartments has been postulated to play a role in the pathogenesis of a number of degenerative disorders (Ho, Magnenat et al. 1998) including OA. Cytokines and growth factors such as IL-1 and TGF-β, as well as mechanical stress, have been shown to stimulate production of ROS in chondrocytes (Stadler, Stefanovic-Racic et al. 1991) (Yamazaki, Fukuda et al. 2003; Loeser 2009) (Aigner, Soder et al. 2007). Evidence has shown that reduced levels of antioxidant enzymes in aged and osteoarthritic cartilage may induce oxidative stress in chondrocytes (Jallali, Ridha et al. 2005). Oxidative stress has been shown to induce DNA damage in chondrocytes and to correlate with chondrocyte senescence (Aigner, Soder et al. 2007). A recent study showed that ROS are involved in reduced proteoglycan synthesis and IGF-1 resistance in chondrocytes mediated by redox-sensitive phosphoinositide-3 (PI-3) kinase-AKT and mitogen-activated protein kinase pathways (MAPK) (Yin, Park et al. 2009).

Degradation of the cartilage matrix, which is a contributor to both the pathogenesis and progression of OA, results in the formation of cartilage lesions.

Pro-inflammatory cytokines, including IL-1, stimulate chondrocyte production of matrix metalloproteinases (MMPs), key degrading enzymes responsible for the breakdown of cartilage matrix (Tetlow, Adlam et al. 2001; Fan, Bau et al. 2005). MMP13 cleaves type II collagen, which is abundant in cartilage, more efficiently than the other types of collagen (Knauper, Lopez-Otin et al. 1996). The importance of MMP13 has been demonstrated in a recent study which showed that MMP13 deficiency prevented cartilage damage in a mouse model of OA (Little, Barai et al. 2009).

Selenium is an essential trace element important for protection against oxidative damage, regulation of thyroid and immune function and, fertility (Rayman 2005; Ryan-Harshman and Aldoori 2005). Selenium functions by incorporating as selenocysteine at the key catalytic domain of selenium-containing proteins, selenoproteins. To date, 25 mammalian selenoproteins have been identified, including antioxidative glutathione peroxidases (GPXs) and thioredoxin reductases (TRs) subfamilies (Kryukov, Castellano et al. 2003). GPXs and TRs catalyze reduction of intracellular peroxide and govern the redox balance in cells (Burk 1990). Gene expression of antioxidant GPXs and thioredoxin-interacting protein (TXNIP) was shown to be down-regulated in OA cartilage in a large scale gene expression study (Aigner, Fundel et al. 2006). In addition, a recent *in vitro* study showed that exposure of chondrocytes to SeMet could block pro-inflammatory interleukin-1 (IL-1) mediated inhibition of cartilage

matrix macromolecule (collagen II, aggrecan) synthesis and MMP1 expression (Andriamanalijaona, Kypriotou et al. 2005). All these lines of evidence highlight potential mechanisms for the protective effect of selenium against OA. We hypothesize that selenium supplementation could increase antioxidative selenoenzyme expression and activity, and block catabolic gene expression induced by IL-1β. In this study, we first established a working system by defining the form and concentration of selenium in SW-1353 cells, a well-characterized chondrosarcoma cell line used for OA study. We further examined whether selenium supplementation could increase GPX and TR gene expression and activities in a SW-1353 cells. In addition, we defined the optimal concentration of IL-1β by measuring gene expression of MMPs in stimulated primary human chondrocytes. Finally, we chose to investigate the protective effect of SeMet on the catabolic effect of IL-1β under our defined conditions.

2.2 Materials and Method

2.2.1 Chondrocyte Isolation and Culture

The samples used for this project were collected under approval of the Duke Internal Review Board (IRB). The IRB deemed these samples surgical waste tissues meeting the definition of research not involving human subjects as described in (45CFR46.102(f)) and was not subject to HIPAA (45CFR164.514(b)) as no information relating to patient identity was obtained with the sample.

Human cartilage specimens were obtained as surgical waste tissues from 5 patients undergoing total knee replacement surgery. Cartilage was harvested from non-lesioned areas, further minced, and subjected to pronase and collagenase digestion to isolate primary chondrocytes, similar to previously published methods (Kuettner, Pauli et al. 1982).The isolated chondrocytes were used for experiments within the first three passages. The SW-1353 chondrosarcoma cell line was obtained from the American Type Culture Collection (ATCC Manassas, VA).

2.2.2 MTT ASSAY

Cell viability was assessed with the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay based on the ability of mitochondria of viable cells to convert soluble MTT into an insoluble purple formazan reaction product. MTT (Sigma) solution (5 mg/ml in DMEM without phenol red) was added to cells in tissue culture for 2 hours. The MTT solution was aspirated, dimethyl sulfoxide (DMSO) was added (200 µl per each well of 12 well plate) to solubilize formazan and detection occurred by addition of 100 µl of the reaction mixture to a 96 well plate and read at 540 nm. MTT assay results for the SW-1353 cell line were derived from 2 independent experiments, performed in duplicate. MTT assay results for primary chondrocytes were derived from 2 independent experiments, performed in triplicate using 2 separate primary chondrocyte cell lines.

2.2.3 RNA Isolation and Real Time RT-PCR

Cell lysates, prepared by using RNeasy Lysis buffer (Qiagen, Valencia, CA), from each experimental condition were homogenized by passage through a QIAshredder spin column (Qiagen, Valencia, CA). The RNA fractions were further isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The isolated total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) for Real Time RT-PCR analysis. The ABI Prism 7000 sequence detection system and relative quantification software (Applied Biosystems, Foster City, CA) were used for real-time analyses. Amplification by real-time RT-PCR used the following Applied Biosystems primer and probe sets: 18S rRNA endogenous control; Hs00829989_gH (GPX1); Hs00702173_sl (GPX2); Hs00173566_ml (GPX3); Hs00157812_ml (GPX4); Hs00699698_ml (GPX6); Hs00233958_ml (MMP1); Hs00968305_ml (MMP3) and Hs00233992_ml (MMP13). Real-time reactions were each performed in triplicate in a final volume of 25 µl.

Raw mRNA expression values were computed by the formula $2^{-\Delta Ct}$ (Livak and Schmittgen 2001) with values normalized to 18S rRNA, where ΔCt represents the difference in Ct (threshold cycle) number of the 18S rRNA gene and the target genes. For GPX gene expression, results were derived from

experiments, performed in triplicate, using SW-1353 cells treated with varying concentrations of SeMet. For gene expression of each MMP, results were derived from experiments, performed in triplicate, using primary human chondrocytes treated with IL-1β with or without SeMet. The relative fold changes in mRNA expression levels of GPXs or MMPs were calculated by the $2^{-\Delta\Delta C_t}$ formula (Livak and Schmittgen 2001), between untreated control cells and treated cells (+/- SeMet and (or) +/- IL-1β). For the purposes of graphical presentation, the relative mRNA level in cells without treatment was set at 100%. Paired t-test between control (no IL-1β, no SeMet) and treated conditions were performed based on the log transformed $2^{-\Delta C_t}$ values unless otherwise specified. P values less than 0.05 were considered significant.

2.2.4 Western Blot Analysis

Chondrocytes from each experimental condition were collected and homogenized in lysis buffer (10 mM HEPES, pH 7.5, 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA and 1 M NaCl), supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO) and phosphatase inhibitors (5 mM PMSF, 10 mM NaF, 25 mM B-glycerophosphate, 0.5 M DTT, 1 mM Na3V04). Whole cell lysates, were further separated by SDS-PAGE, and transferred to nitrocellulose for immunoblotting. Membranes were blocked with 5% BSA in TBS/0.1% Tween 20 (TBS-T). Polyclonal primary antibodies against GPX1, GPX4 and TR1

were obtained from Abcam (Cambridge, MA) and used at 1:1000 dilution. A monoclonal antibody against α-tubulin (Sigma) was used as a normalization control at 1:10,000 dilution. Anti-rabbit and anti-mouse IgG-HRP (Jackson ImmunoResearch, West Grove, PA) secondary antibodies were used at a 1:5,000 dilution. The resulting films were scanned using CanoScan LiDE 70 (Canon, Lake Success, NY). Signal intensity was quantified by Image J analysis of Western blots (as described in 2A) with normalization to the internal control, α-tubulin.

2.2.5 GPX Activity Assay

Cellular GPX activity in each experimental condition was determined with Glutathione Peroxidase Assay Kit (Cayman, Ann Arbor, MI) according to the manufacturer's protocol. This assay is based on the method of Paglia and Valentine (Paglia and Valentine 1967), in which the activity is determined by following the rate of NADPH oxidation at 340 nm using cumene hydroperoxide as substrate. The assay reactions were each performed in a final volume of 200 µl on a 96 well platform for reading at 340 nm. GPX assay results for the SW-1353 cell line were derived from 2 independent experiments, performed in triplicate. One unit of activity was defined as one nmole of NADPH oxidized per min at 25^{o}C. The results were expressed as unit per µg of protein. Paired t-test was

performed between control (no SeMet) and treated conditions. P values less than 0.05 were considered significant.

2.2.6 TR Activity Assay

Cellular TR activity in each experimental condition was determined with Thioredoxin Reductase Assay Kit (Sigma, St. Louis, MO) according to the manufacturer's protocol. The assay is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) in the presence of NADPH, which resulted in an increase in absorbance at 412 nm (Holmgren and Bjornstedt 1995). Each assay reaction was performed in a final volume of 200 µl in a 96 well plate platform for reading at 412 nm. TR assay results for the SW-1353 cell line were derived from 2 independent experiments, performed in triplicate. One unit of activity is equal to an increase of absorbance at 412 nm of 1.0 per minute per ml at pH 7.0 at 25^{o}C. The results were expressed as unit per µg of protein. Paired t-test was performed between control (no SeMet) and treated conditions. P values less than 0.05 were considered significant.

2.2.7 Total Specific MMP Activity Assay

Primary chondrocytes were seeded at a density of 2.0 x10^5 cells per well on a 12-well plate. Chondrocytes were cultured in phenol red-free medium for 24 hours in the absence (control) or presence of 0.5 μM SeMet, followed by 24 hour co-treatment without IL-1β or with 100 pg/ml IL-1β. The relative number of cells in each well was estimated by cell viability assay for normalization of MMP activity. The culture media was measured for total specific MMP activity. The media samples were activated with 1 mM p-amino phenylmercuric acetate (AMPA) at 37℃ for 2 hour. The total specific MMP activity fr om the treated culture media was based on a fluorescence based assay modified from the protocol described previously (Wilusz, Weinberg et al. 2008). Media samples were twofold diluted in assay buffer consisting of 200 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 10 μM $ZnSO_4$, 0.01% Brij 25, pH 7.5 and 20 μM quenched fluorogenic MMP13 peptide substrate Dab-Gly-Pro-Leu-Gly-Met-Arg-Gly-Lys-Flu (Sigma-Aldrich custom order) (Deng, Bickett et al. 2000; Rasmussen, Yeung et al. 2004). The media samples were run in duplicate, with one replicate incubated with peptide alone and the other incubated with peptide and 10 mM ethylenediaminetetraacetic acid (EDTA, for inhibiting MMP activity). Samples were incubated in the dark at 37℃ for 2 hours. Fluorescence was measured at 485 nm excitation and 535 nm emission. The total specific MMP activity was obtained by the difference in measured fluorescence between medium samples incubated with or without

EDTA (this adjustment removes non-specific activity or background) normalized by relative cell number determined by cell viability assay. For the purposes of graphical presentation, the relative total specific MMP activity of media samples from cells without treatment was set at 100%. Paired t-test was performed between control (no SeMet) and treated conditions. P values less than 0.05 were considered significant.

2.3 Results

2.3.1 Cytotoxic Effect of Selenite and SeMet in SW-1353 and Primary Chondrocytes

For cell toxicity assays we initially compared two forms of selenium, organic selenomethionine (SeMet) and inorganic selenite, in SW-1353 cells. Based on the MTT toxicity assay, SeMet showed no evidence of cellular toxicity at concentrations up to 1 µM in SW-1353 cells (Figure 2.1A) while selenite showed cell toxicity beyond 1 µM. We further demonstrated that SeMet at a concentration of 0.5 µM was non toxic when treated alone or in combination with IL-1β in primary chondrocytes (Figure 2.1B).

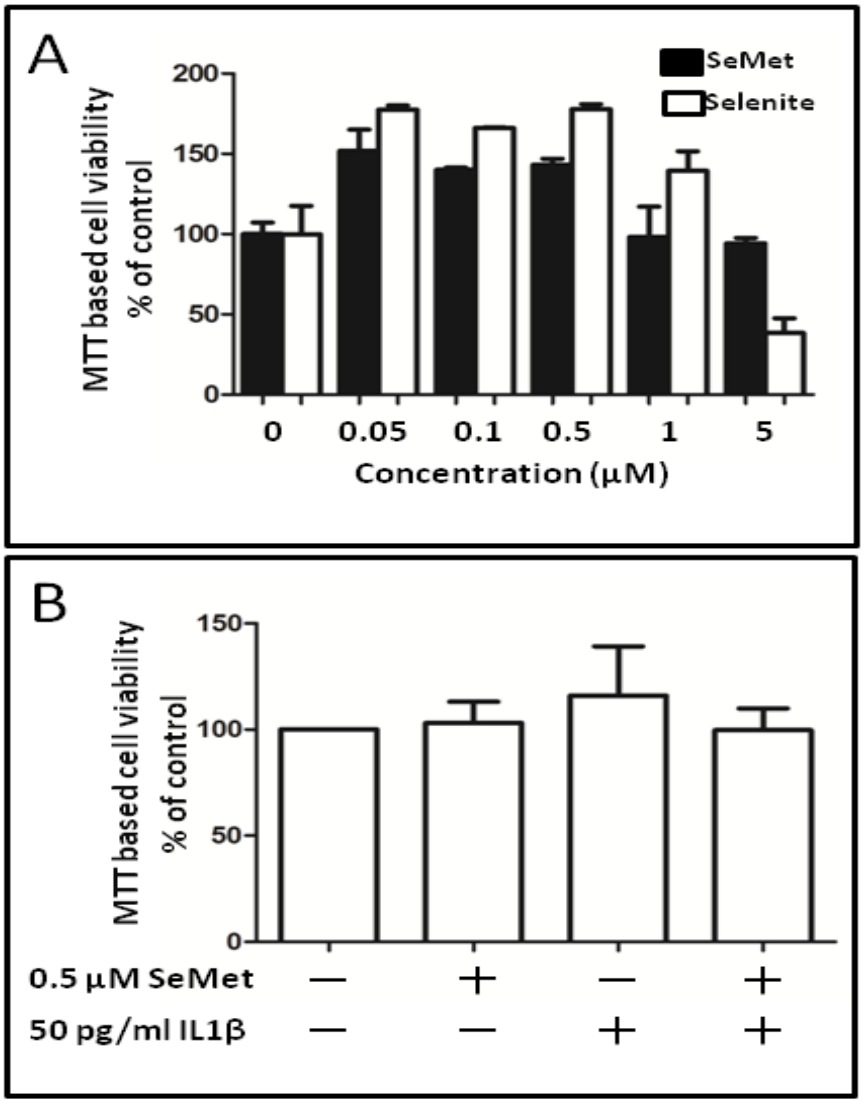


Figure 2.1 MTT cell toxicity assay for optimization of *in vitro* chondrocyte culture conditions. Effects of selenium on cell viability were assessed with the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. **(A)** SW-1353 cells were cultured with different concentrations (0 µM to 5 µM) of selenite or selenomethionine (SeMet) for 48 hours. **(B)** Primary human chondrocytes were cultured for 24 hours in the absence (control) or presence of 0.5 µM SeMet, followed by 24 hours co-treatment without or with 50 pg/ml IL-1β. Values shown are mean and SEM of cell viability as a percentage of control (set at 100%).

2.3.2 Effect of Serum Starvation on Selenoprotein Marker Expression and Cell Viability in SW-1353 Cells

In order to minimize the effect of residual trace amounts of selenium from the serum used in cell culture, a serum (or selenium) depletion study was performed to determine the length of time needed for the cell to exhaust cellular selenium storage. The cellular selenium status was monitored by measuring protein expression of GPXs in SW-1353 cells, as their expression levels have been shown to be sensitive to selenium status (Bermano, Nicol et al. 1996; Hesketh 2004), and have been used to evaluate the effect of selenium supplementation (Brown, Pickard et al. 2000). The protein expression level of GPX1 was reduced by approximately 60% and 75% at 24 and 48 hours, respectively, after serum depletion (Figure 2.2). Similarly, the GPX4 protein level was decreased by approximately 50% and 70% at 24 hours and 48 hours, respectively, after serum depletion. In contrast, both GPX1 and GPX4 protein levels were surprisingly increased at 72 hours after serum depletion. Under the same condition, a decrease in cell number became obvious between 48 hours and 72 hours of serum deprivation in cell viability test (Figure 2.3). These data suggested the optimal time of serum deprivation is between 24 hours and 48 hours.

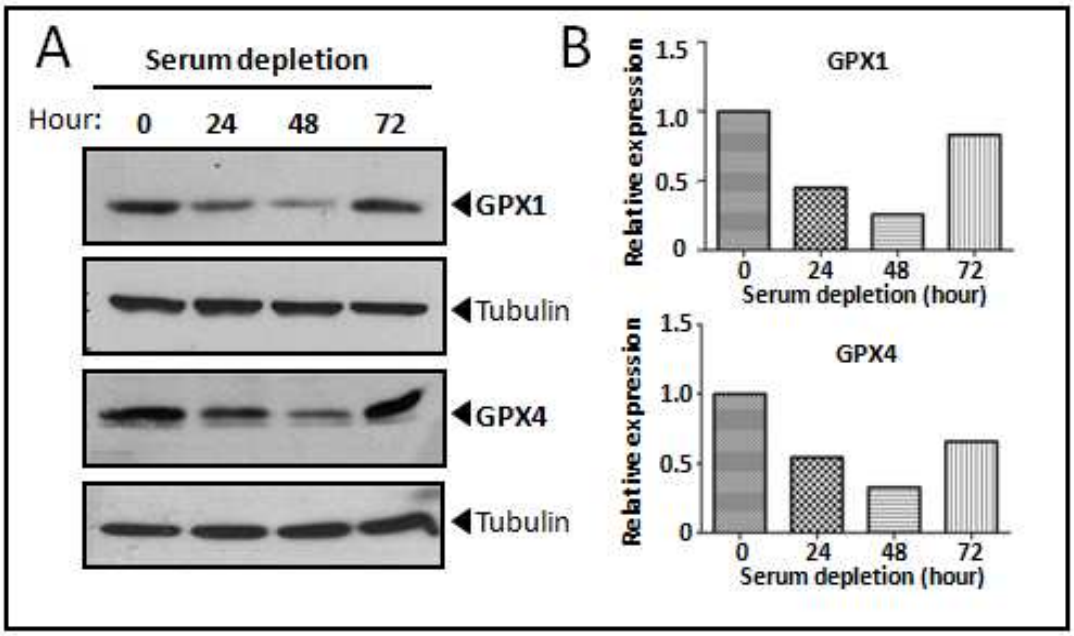


Figure 2.2 Effect of serum starvation on selenoprotein marker expression in SW-1353 cells. (A) Western blot analysis. SW-1353 cells were serum starved for 0 hour, 24 hours, 48 hours and 72 hours. Equal amounts of total cell lysate were separated by SDS-PAGE and analyzed by Western blot for the GPX1 and GPX4 proteins. α-tubulin (bottom row) was used as a control for normalization. **(B)** Quantification of the effect of serum starvation on GPX1 and GPX4 protein levels in SW-1353 cells. Signal intensity was quantified by Image J analysis of Western blots (as described in 2A) with normalization to the internal control, α-tubulin. Values shown are relative protein expression as a percentage of control (1 at time=0)

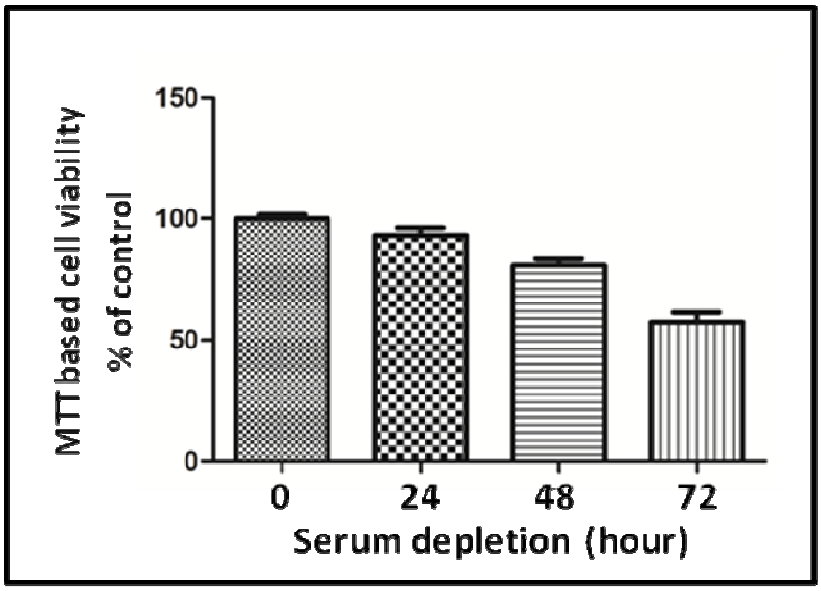


Figure 2.3 Effect of serum starvation on cell viability of SW-1353 cells
Effects of selenium on cell viability were assessed with the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. SW-1353 cells were serum starved for 0 hour, 24 hours, 48 hours and 72 hours. Values shown are mean and SEM of cell viability as a percentage of control (set at 100%).

2.3.3 Effect of Se Supplementation on GPX and TR Expression and Activity in SW-1353 Cells

Forty eight hour treatment with SeMet increased mRNA levels of all selenium-containing peroxidases in a dose-dependent manner except GPX6, which was undetectable in SW-1353 cells. The effect of SeMet on mRNA expression of GPXs peaked at 0.5 µM: GPX1-3, upregulated approximately 1 to 1.5-fold while GPX4 upregulated approximately 0.5-fold (Figure 2.4). SeMet-increased protein expression of GPX1 and GPX4 was further confirmed in immunoblot analysis, in which the protein levels of GPX1 and GPX4 were found to increase significantly up to 4-fold and 3-fold, respectively, even at the lowest concentration of SeMet of 0.25 µM (Figure 2.5). In order to examine if the increased GPXs protein levels were assoicated with concomitant increases in activity, we performed GPX activity assays. The cellular GPX activity was significantly increased 1 to 1.5-fold by SeMet at concentrations of 0.25 µM and 0.5 µM ($P < 0.01$) (Figure 2.6).

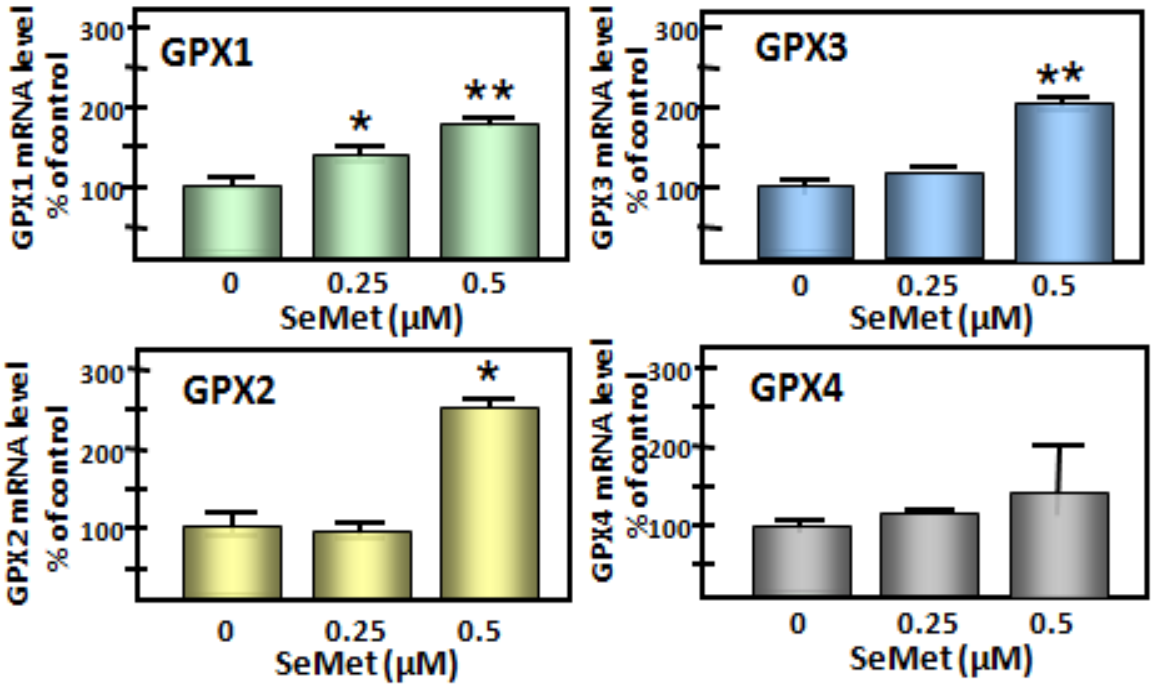


Figure 2.4 SeMet increases GPX1-4 mRNA expression in SW-1353 cells. SW-1353 cells were treated with increasing concentrations from 0 µM to 0.5 µM of SeMet for 48 hours. Gene expression for GPXs was determined by RT-PCR normalized to 18S rRNA (average of triplicates). * P< 0.05; ** P< 0.01 compared to untreated control (0 µM).

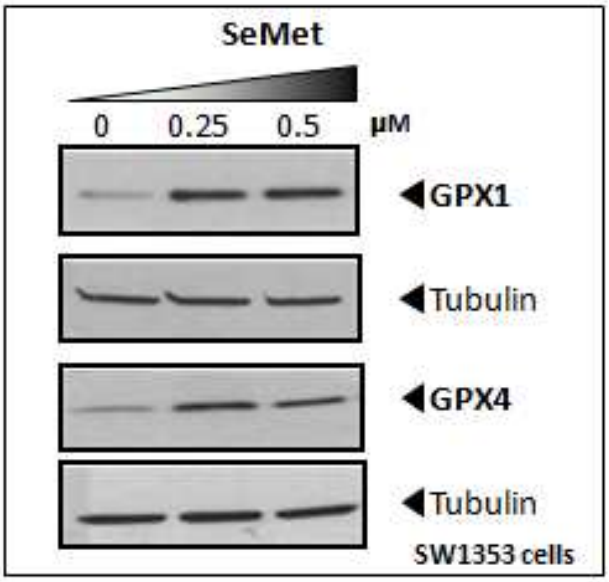


Figure 2.5 SeMet increases GPX1 and GPX4 protein expression in SW-1353 cells. SW-1353 cells were treated with increasing concentrations from 0 μM to 0.5 μM of SeMet for 48 hours. Equal amounts of total cell lysate were separated by SDS-PAGE and analyzed by Western blot for GPX1 and GPX4 proteins. α-tubulin (bottom row) was used as a control for normalization.

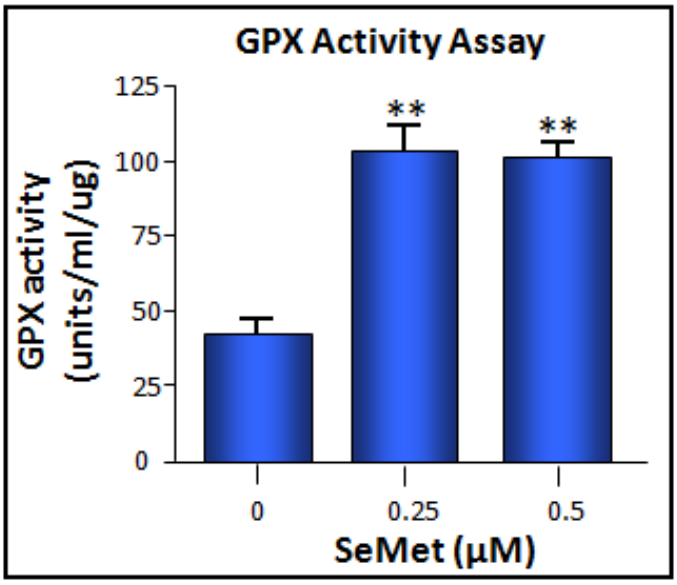


Figure 2.6 SeMet increases cellular GPX activity in SW-1353 cells. SW-1353 cells were treated with increasing concentrations from 0 µM to 0.5 µM of SeMet for 48 hours. GPX activity was determined by measuring the rate of NADPH oxidation at 340 nm in the presence of the substrate cumene peroxide. One unit of activity was defined as one nmole of NADPH oxidized per min. Results were expressed as unit per µg of protein. Values shown are mean and SEM of GPX activity (average of triplicates for 2 independent experiments). ** P <0.01 compared to untreated control (0 µM).

Similar to the effect on GPX expression, forty eight hour treatment with SeMet increased protein levels of TR1 by 1 to 2-fold at 0.25 μM and 0.5 μM. (Figure 2.7A). A further study was performed to examine the overall change in intracellular TR activity that could be affected by the SeMet treatment. The result showed that the cellular TR activity was increased 2-3-fold and 1-1.5-fold by SeMet at 0.25 μM and 0.5 μM, respectively (Figure 2.7B).Taken together, these result demonstrated that expression and activity of GPXs and TRs could be increased by SeMet in our *in vitro* system.

2.3.4 Concentration-dependent Effect of IL-1β on MMP Gene Expression in Primary Chondrocytes

In order to define the optimal concentration range of IL-1β in our study, we examined expression of MMPs, well-studied catabolic genes stimulated by IL-1β, in response to different concentrations of IL-1β. Treatment for twenty four hours with IL-1β increased all MMP mRNA levels in a concentration-dependent manner (Figure 2.8). At the concentrations of 10 pg/ml and 100 pg/ml, IL-1β increased MMP1 mRNA levels by 23-fold and 140-fold, respectively; MMP3 mRNA levels by 15-fold and 23-fold, respectively; and MMP13 mRNA levels by 4-fold and 7-fold respectively compared to untreated control. At the concentration of 1 ng/ml,

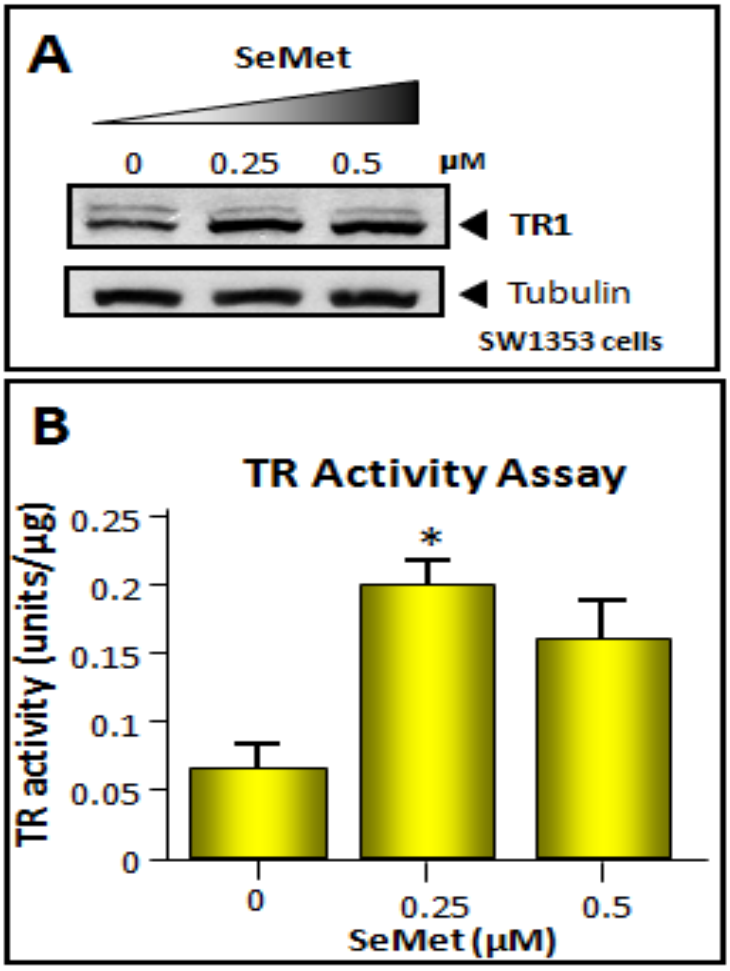


Figure 2.7 SeMet increases TR1 protein expression and TR activity in SW-1353 cells. SW-1353 cells were either untreated or treated with 0.25 μM or 0.5 μM of SeMet for 48 hours **(A)** SeMet increased TR1 protein level in SW-1353 cells. Equal amounts of total cell lysate were separated by SDS-PAGE and analyzed by Western blot for TR1 protein. α-tubulin (bottom row) was used as a control for normalization. **(B)** SeMet increased cellular TR activity in SW-1353 cells. The assay is based on the reduction of DTNB in the presence of NADPH which resulted in an increase in absorbance at 412 nm. One unit of activity is equal to an increase of absorbance at 412 nm of 1.0 per minute per ml at pH 7.0 at 25^{o}C. The results were expressed as unit per μg of protein. Values shown are mean and SEM of TR activity (average of triplicates for 2 independent experiments). * P< 0.05, compared to untreated control (0 μM)

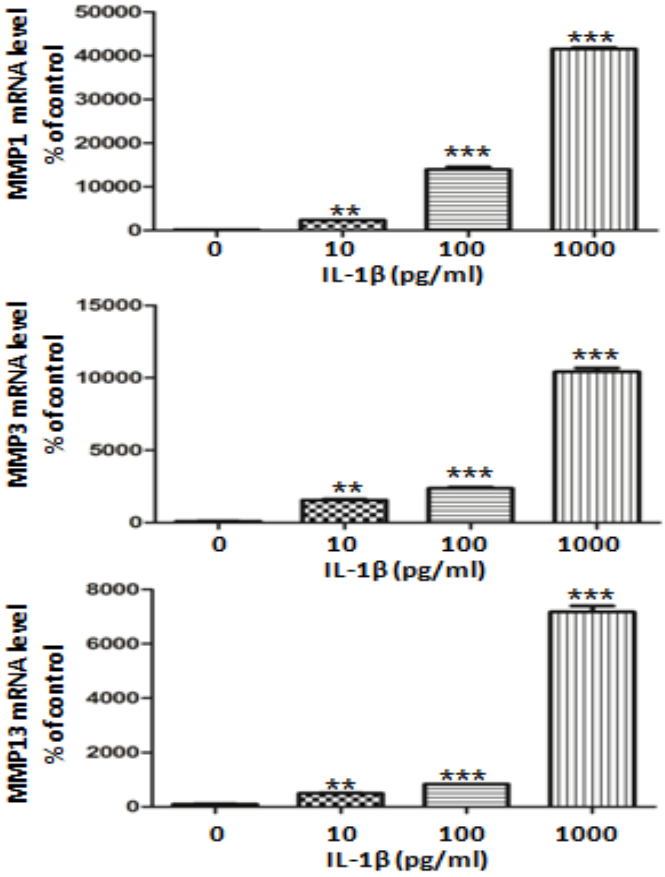


Figure 2.8 IL-1β increases MMPs mRNA levels in primary human chondrocytes. Primary human chondrocytes were cultured for 24 hours in the absence of serum, followed by 24 hour treatment without or with IL-1β at 10 pg/ml, 100 pg/ml and 1000 pg/ml. Gene expression for MMP1, MMP3, MMP13 was determined by RT-PCR normalized to 18S rRNA (average of triplicates) ** P< 0.01; ***p<0.001, compared to untreated control (0 µM).

IL-1β dramatically increased MMP1, MMP3 and MMP13 mRNA level by approximately 400-fold, 100-fold and 70-fold, respectively. These data suggested that the optimal concentration of IL-1β in this system is between 10 pg/ml and 100 pg/ml.

2.3.5 Effect of SeMet on IL-1β-Increased MMPs Gene Expression and Total Specific MMP Activity

Twenty four hour treatment with 100 pg/ml IL-1β increased MMP1, MMP3 and MMP13 mRNA levels by approximately 6-fold, 5-fold and 14-fold, respectively. SeMet alone decreased basal MMP1 mRNA level but only slightly decreased basal MMP3 and MMP13 mRNA levels. Twenty four hour pretreatment with SeMet repressed both IL-1β-induced MMP1 and MMP13 steady state mRNA level by approximately 15% ($p<0.05$) and 44% ($p<0.01$), respectively (Figure 2.9). In contrast, SeMet pretreatment had no significant effect on IL-1β-induced MMP3 mRNA level. We further examined the effect of SeMet on total specific MMP activity. SeMet pretreatment inhibited IL-1β-induced total specific MMP activity by approximately 21% ($p<0.05$) at 100 pg/ml IL-1β (Figure 2.10).

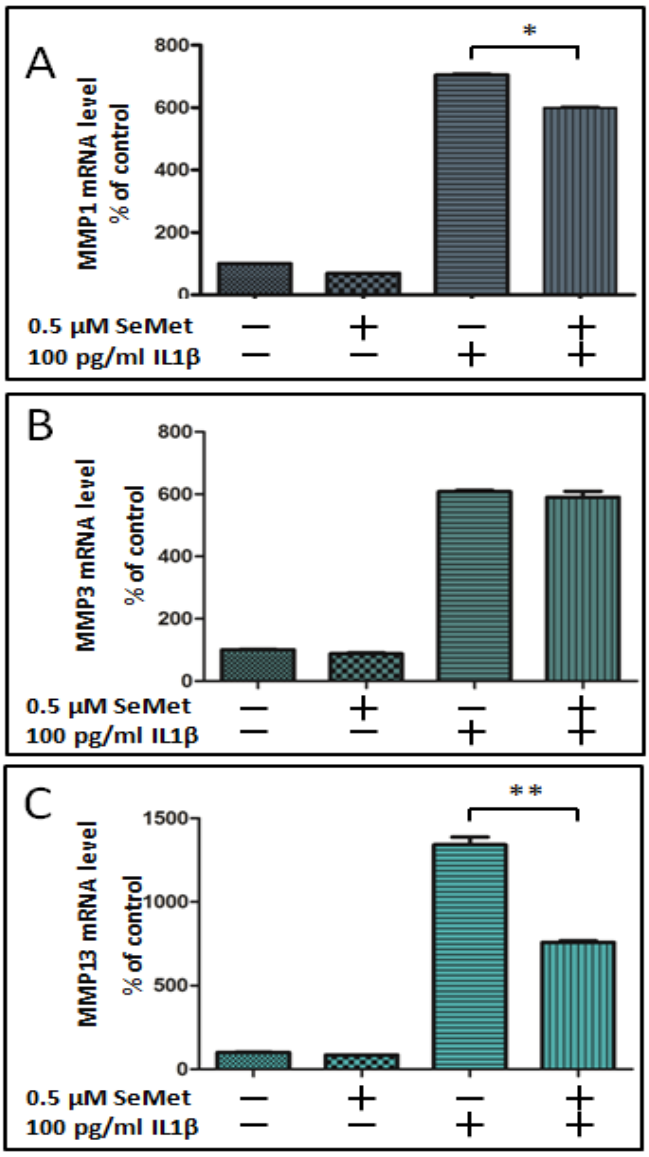


Figure 2.9 Effect of SeMet on IL-1β-induced MMP gene expression in primary human chondrocytes. Primary human chondrocytes were cultured for 24 hours in the absence (control) or presence of 0.5 µM SeMet, followed by 24 hour co-treatment without IL-1β (control) or with100 pg/ml IL-1β. Gene expression for MMP1, MMP3 and MMP13 (**A, B and C**) was determined by RT-PCR normalized to 18S rRNA (average of triplicates). Values shown are mean and SEM of individual MMP as a percentage of control (set at 100%). *P<0.05, **P<0.01

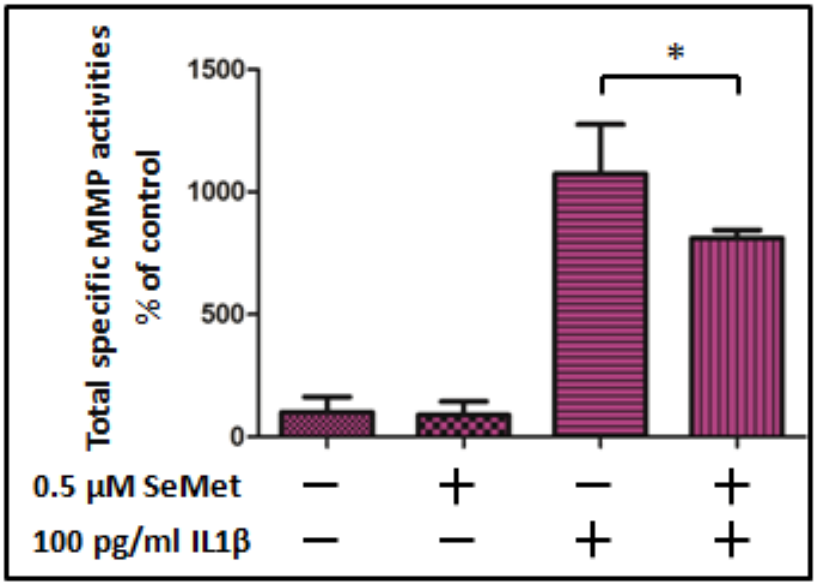


Figure 2.10 SeMet inhibits IL-1β-induced total specific MMP activity in primary human chondrocytes. Primary human chondrocytes were cultured for 24 hours in the absence (control) or presence of 0.5 µM SeMet, followed by 24 hour co-treatment without IL-1β (control) or with 100 pg/ml IL-1β. Total specific MMP activity in the corresponding media was determined using a quenched fluorescent substrate and normalized to the relative number of the cells (average of triplicates). Values shown are mean and SEM as a percentage of control (set at 100%). *P<0.05

2.4 Discussion

Selenium has been called a “two-faced element” (Koller and Exon 1986). Selenium is an essential nutrient for the human body if it is in trace amounts. However, it becomes a toxin when it is in excess. This toxic but essential characteristic of selenium also has been shown in this study (Figure 2.1A). Low concentrations of selenium, in both organic (SeMet) and inorganic (selenite) forms, increased cell viability of SW-1353 cells, while high concentrations of Se reduced cell viability. SeMet was less toxic compared to selenite at high concentration. This is consistent with data showing that selenite caused a more severe toxic effect (during selenosis) at earlier time points compared to SeMet when swine consumed a high selenium diet (Kim and Mahan 2001). We subsequently chose the concentration of SeMet at 0.5 µM based on the cell viability test. This concentration was also shown to have physiological relevance that it is equivalent to concentrations found in normal and OA human synovial fluid (Yazar, Sarban et al. 2005).

A measureable decrease in protein expression levels of GPX1 and GPX4 in SW-1353 cells was found at 24 to 48 hours after serum starvation (Figure 2A). This was consistent with the finding shown in other studies that GPX expression levels are sensitive to selenium status (Bermano, Nicol et al. 1996; Hesketh 2004). The reduced protein expression levels of GPX1 and GPX4 could be a result of their mRNA instability through nonsense-mediated mRNA decay (NMD)

under selenium deficiency conditions, as reported previously (Sun, Li et al. 2001). Unexpectedly, the protein level of both GPX1 and GPX4 increased at 72 hours after serum starvation (selenium depletion). This change in GPX1 and GPX4 protein expression correlated with reduced cell viability at 72 hours after serum starvation (shown in Figure 2B). The increased GPX1 and GPX4 protein expression levels at 72 hours could be a stress response to the toxic effect of prolonged serum starvation.

Overproduction of ROS in chondrocytes in response to various stimuli (Stadler, Stefanovic-Racic et al. 1991; Yamazaki, Fukuda et al. 2003; Aigner, Soder et al. 2007; Loeser 2009) has been proposed to be involved in the pathogenesis of OA (Ho, Magnenat et al. 1998). Our data showed that SeMet supplementation increased both GPXs and TRs gene expression and activity in SW-1353 cells. This highlights the possibility that selenium may mediate its protective effect through GPX and TR against oxidative stress especially in chondrocytes. Previous studies demonstrated GPX and TR activities can alter redox sensitive pathways such as NFkB and MAPK pathway (Brigelius-Flohe, Friedrichs et al. 1997; Rundlof and Arner 2004) and modulate the inflammatory response induced by cytokines including interleukin-1 (Brigelius-Flohe, Friedrichs et al. 1997). We chose a concentration range of 10 pg/ml to100 pg/ml as optimal concentrations for IL-1β based on increased gene expression of MMPs. These concentrations are physiologically relevant (equivalent to concentrations in

human OA synovial fluid) (Westacott, Whicher et al. 1990; Kahle, Saal et al. 1992). In addition, SeMet inhibited IL-1β-induced catabolic MMPs gene expression and total specific MMP activity in primary chondrocytes in our conditions (Figure 2.9 and 2.10). This is consistent with a previous study which also demonstrated the inhibitory effect of selenium on MMP1 gene expression (Andriamanalijaona, Kypriotou et al. 2005). Given the deleterious role of MMP in cartilage degradation, our data suggest selenium may be protective through neutralizing the catabolic effect of IL-1β. Based on our data, it is tempting to speculate that selenium increases GPX and TR expression and activity and in turn alters IL-1β signaling pathway(s), which activate MMP gene expression. Further experiments are needed to study this possibility.

In summary, we established our model system and chose a concentration of 0.5 μM for SeMet and a concentration range of 10-100 pg/ml for IL1β for the rest of our study. Our data showed SeMet increased both GPX and TR gene expressions and activities in SW-1353 cells. SeMet inhibited IL-1β-induced MMP1 and MMP13 gene expression and total MMP specific activity. These results suggest selenium may exert a protective effect through regulation of antioxidative enzyme expression and neutralizing catabolic effect of IL-1β.

CHAPTER 3

Selenomethionine Inhibits IL-1β-Inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase 2 (COX2) Expression in Primary Human Chondrocytes

Reprinted from Osteoarthritis and Cartilage. Cheng AW, Stabler TV, Bolognesi M, Kraus VB. Selenomethionine inhibits IL-1β induced nitric oxide (iNOS) and cyclooxygenase 2 (COX2) in primary human chondrocytes, in press, 2010 with permission from Elsevier.

3.1 Introduction

Selenium is an essential trace element involved in several key metabolic activities: protection against oxidative damage, regulation of immune and thyroid function, and fertility (Rayman 2000; Ryan-Harshman and Aldoori 2005). Recently, a growing interest in the potentially protective role of selenium in osteoarthritis (OA) has been generated based on epidemiology (Allander 1994; Jordan, Fang et al. 2005; Jordan, Fang et al. 2007), genetic (Meulenbelt, Bos et al. 2010) and transgenic animal studies (Downey, Horton et al. 2009). Profound selenium deficiency is associated with the severe osteoarthropathy known as Kashin-Beck Disease that affects individuals in China (as many as 7 million) and neighboring regions (Allander 1994). Evidence for a role for selenium in OA comes from a large population-based study in the US in which a low but non-deficiency level of selenium was shown to be associated with OA presence and severity (Jordan, Fang et al. 2005; Jordan, Fang et al. 2007). Moreover, a recent genetic study showed that a variant of *Deiodinase 2* (*DIO2*), which encodes a selenoprotein involved in thyroid hormone activation, is associated with risk for developing OA (Meulenbelt, Min et al. 2008). While the exact mechanism is still unclear*, DIO2* is hypothesized to play a role in bone remodeling in OA progression (Meulenbelt, Min et al. 2008). In mice, conditional knockout of the selenocysteine tRNA gene*,* which is required for incorporation of selenium into selenoproteins, results in skeletal abnormalities and severe chondronecrosis of

articular cartilage resembling Kashin-Beck Disease, as well as chondronecrosis of auricular and tracheal cartilages (Downey, Horton et al. 2009).

In vitro studies have also suggested a protective effect of selenium. For instance, selenium alters iNOS and COX2 gene expressions in response to lipopolysaccharide (LPS) stimulation in cultured macrophages (Prabhu, Zamamiri-Davis et al. 2002; Vunta, Belda et al. 2008). Lack of selenium *in vitro* has been associated with elevated Prostaglandin E_2 (PGE_2) production in calcium ionophore-stimulated endothelial cells (Cao, Reddy et al. 2000) and LPS-induced macrophages (Zamamiri-Davis, Lu et al. 2002). A recent study showed that exposure of chondrocytes *in vitro* to selenomethionine (SeMet) could block IL-1-mediated inhibition of cartilage matrix macromolecule (collagen II and aggrecan) synthesis (Andriamanalijaona, Kypriotou et al. 2005). However, in the same study, SeMet did not have significant effects on iNOS or COX2 gene expression in the presence of high doses of IL-1β in bovine chondrocytes.

Taken together, these studies suggest an important role for selenium in maintaining normal cartilage metabolism and potentially preventing OA. A better understanding of the mechanisms underlying these selenium-mediated protective effects could lead to the development of novel therapeutic approaches for the prevention and management of OA progression. We hypothesize that SeMet could block proinflammatory gene expression induced by physiological doses of IL-1β. In this study we chose to investigate potential mechanisms of selenium-

mediated protective effects using physiological rather than supra-physiological doses of IL-1β as used in the majority of prior experiments (Thomas, Thirion et al. 2002; Lazzerini, Capecchi et al. 2004; Andriamanalijaona, Kypriotou et al. 2005; Panico, Cardile et al. 2005; Zhou, Liu et al. 2008). Under these conditions we investigated the magnitude of selenium effects and the potential signaling pathways involved in primary human chondrocytes.

3.2 Materials and Methods

3.2.1 Chondrocyte Isolation and Culture

The samples used for this project were collected under approval of the Duke IRB. The IRB deemed these samples surgical waste tissues meeting the definition of research not involving human subjects as described in (45CFR46.102(f)) and was not subject to HIPAA (45CFR164.514(b)) as no information relating to patient identity was obtained with the sample. Articular cartilage samples were obtained as surgical waste tissues from 19 patients undergoing total knee replacement surgery [mean age, 61.8 +/- 7.7 years]. Cartilage was harvested from non-lesional areas, further minced, and subjected to pronase and collagenase digestion to isolate primary chondrocytes, similar to previously published methods (Kuettner, Pauli et al. 1982). Isolated chondrocytes were used within the first three passages. The SW-1353 chondrosarcoma cell

line was obtained from the American Type Culture Collection (ATCC Manassas, VA).

IL-1β (R & D systems, Minneapolis, MN), concentrations of 10 pg/ml and 50 pg/ml were chosen on the basis of evidence for physiological relevance (equivalent to concentrations in human OA synovial fluid) (Westacott, Whicher et al. 1990; Kahle, Saal et al. 1992). Selenomethionine (SeMet) (Sigma, St Louis, MO), an organic form of selenium, was chosen as it is the primary dietary source of Se for human (Schrauzer 2000). A concentration of 0.5 μM SeMet was chosen for chondrocyte cultures based on evidence of physiological relevance (equivalent to concentrations in normal and OA human synovial fluid (Yazar, Sarban et al. 2005)).

3.2.2 RNA Isolation and Real Time RT-PCR

Cell lysates, prepared by RNeasy Lysis Buffer (Qiagen Valencia, CA) from each experimental condition, were first homogenized by passing them through a QIAshredder spin column (Qiagen, Valencia, CA). The total DNA and RNA fractions were further isolated using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The isolated total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) for Real Time RT-PCR analysis. The ABI Prism 7000 sequence detection system and relative

quantification software (Applied Biosystems, Foster City, CA) were used for real-time analyses. The amplification for real-time RT-PCR used the following Applied Biosystems primer and probe sets: 18S rRNA endogenous control, Hs01075527_m1(iNOS), and Hs01573474_g1(COX2). The real-time reactions were each performed in triplicate in a final volume of 25 µl.

3.2.3 mRNA Quantification and Statistical Analysis

Raw mRNA expression values were computed by $2^{-\Delta Ct}$ formula (Livak and Schmittgen 2001) with values normalized to 18S rRNA, where ΔCt represents the difference in Ct (threshold cycle) number of the 18S rRNA gene and the iNOS or COX2 genes. Results were derived from a total of 6 independent experiments for each dose of IL-1β for COX2 and iNOS respectively, performed in triplicate, using a total of 13 separate primary chondrocyte cell lines. The relative fold changes in mRNA expression levels of iNOS and COX2 were calculated by the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen 2001), between three different treatments (SeMet, IL-1β, SeMet and IL-1β treatment) and control without treatment (no SeMet, no IL-1β). For the purposes of graphical presentation, the relative mRNA level in cells without treatment was set at 100%.

Raw mRNA expression data were evaluated by two tailed Wilcoxon matched pairs test comparing subgroups (n=6 in each group from 13 separate cell lines): 1) the control group and SeMet pretreated group, 2) the IL-1β treatment group

and IL-1β with SeMet pretreatment group, and 3) the control group and IL-1β treatment group. The nonparametric Wilcoxon matched pairs test was chosen as it is appropriate for comparing two paired groups allowing for the non-symmetrical distribution of the raw mRNA expression data (Motulsky 1990).

3.2.4 Nitrite and PGE_2 Assays

Nitrite (NO2-, one of the stable end products of NO) concentrations were determined by chemiluminescence using an Ionics/Sievers nitric oxide analyzer (NOA 280, Sievers Instruments, Boulder, CO), per the manufacturer's instructions. Potassium iodide in acetic acid was used as a reductant in this analysis because of its specificity for nitrite. Nitrite concentrations were determined by nitrite standards prepared from sodium nitrite (Sigma, St Louis, MO) and normalized to total DNA isolated from the corresponding chondrocytes. The total DNA concentration was quantified by Nanodrop 1000 (Thermo Scientific, Wilmington, DE).

The PGE_2 concentration of the collected culture medium was determined using a competitive enzyme immunoassay-based Prostaglandin E2 Parameter Assay Kit (R&D Systems, Minneapolis, MN). The data were normalized to total DNA isolated from the corresponding chondrocytes. For samples with undetectable PGE_2 levels (from the untreated control groups and the SeMet-

pretreated alone groups), a value was assigned (13.5 pg/ml) which was equal to half the lowest detection limit of the kit.

3.2.5 Nitrite and PGE_2 concentration Quantification and Statistical Analysis

Results of nitrite and PGE_2 analyses were derived from a total of 6 independent experiments for each dose of IL-1β for nitrite and PGE_2 respectively, using a total of 14 separate primary chondrocyte cell lines. For the purposes of graphical presentation, the fold changes in nitrite and PGE_2 concentrations were calculated normalized to total DNA. The relative concentration in cells without treatment (no SeMet, no IL-1β) was set at 100%. Nitrite and PGE_2 concentration data were evaluated by two tailed Wilcoxon matched pairs test comparing subgroups (n=6 in each group from 14 separate cell lines): 1) the control group and SeMet-pretreated group, 2) the IL-1β treatment group and IL-1β with SeMet pretreatment group, and 3) the control group and IL-1β treatment group. All analyses were performed using GraphPad version 5.0 (GraphPad Software, LA Jolla, CA). The nonparametric Wilcoxon matched pairs test was chosen as it is appropriate for comparing two paired groups allowing for the non-symmetrical distribution of the nitrite and PGE_2 data (Motulsky 1990).

3.2.6 Western Blot Screening of Signal Transduction Proteins

Chondrocytes from each experimental condition were collected and homogenized in lysis buffer (10 mM HEPES, pH 7.5, 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA and 1 M NaCl), supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO) and phosphatase inhibitors (5 mM PMSF, 10 mM NaF, 25 mM B-glycerophosphate, 0.5 M DTT, 1 mM Na3V04). Whole cell lysates, were further separated by SDS-PAGE, and transferred to nitrocellulose for immunoblotting. Membranes were blocked with 5% BSA in TBS/0.1% Tween 20 (TBS-T).

Polyclonal primary antibodies against Phospho-p38 MAPK (Thr180/Tyr182), Total p38 MAPK, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Phospho-SAPK/JNK (Thr183/Tyr185), Phospho-IKKα/β (Ser176/180) and Phospho-NFkB p65(Ser536) were obtained from Cell Signaling Technology (Danvers, MA) and used at 1:1000 dilution. A monoclonal antibody against α-tubulin (Sigma) was used as a normalization control at 1:10,000 dilution. Anti-rabbit and anti-mouse IgG-HRP (Jackson ImmunoResearch, West Grove, PA) secondary antibodies were used at a 1:5,000 dilution. The resulting films were scanned using CanoScan LiDE 70 (Canon, Lake Success, NY) and the band intensities were quantified using Adobe Photoshop CS and Image J (National Institutes of Health, Bethesda, MD). The mean and SEM intensities of the phosphosignaling proteins were normalized to the internal protein control (α-tubulin).

3.2.7 Phosphosignaling Protein Quantification and Statistical Analysis

Raw intensity data of phosphosignaling proteins were normalized to the internal protein control (α-tubulin). Results were generated for three time points 5, 30 and 45 minutes in the presence and absence of SeMet, and were derived from 3 independent experiments from 3 separate primary chondrocyte cell lines. The total area under each curve (from 5 to 45 minutes) was determined. The phosphosignaling protein intensities were evaluated by two-tailed Wilcoxon matched pairs test comparing the response to IL-1β induction at 5, 30 and 45 minutes between control (n=9, without Se pretreatment) and the selenium pretreated (n=9, 0.5 μM for the prior 24 hours).The Wilcoxon matched pairs test was chosen as it is appropriate for comparing two paired groups allowing for the non-symmetrical distribution of the phosphosignaling data.

3.3 Results

3.3.1 Inhibitory Effect of SeMet on IL-1β-Induced iNOS Gene Expression and Nitrite Production

Twenty four hour treatment with IL-1β at 10 pg/ml and 50 pg/ml significantly induced iNOS mRNA (approximately 70 fold and 630 fold respectively) compared to the control without IL-1β (Figures 3.1A and 3.1B). SeMet alone had no effect on the basal iNOS mRNA level. Twenty four hour pretreatment with SeMet significantly repressed IL-1β-induced iNOS steady state

mRNA level by approximately 54% at 10 pg/ml IL-1β (P=0.031), and by approximately 31% at 50 pg/ml IL-1β (P=0.031). We further examined the effect of SeMet on nitric oxide (NO), the enzymatic product of iNOS. Because of the short half-life of reactive NO, we measured nitrite ($NO2^-$), which is a stable downstream product of NO. SeMet pretreatment inhibited IL-1β-induced nitrite production, by 47% at 10 pg/ml IL-1β (P=0.031), and by 19% at 50 pg/ml IL-1β (P=0.031) (Figures 3.1C and 3.1D).

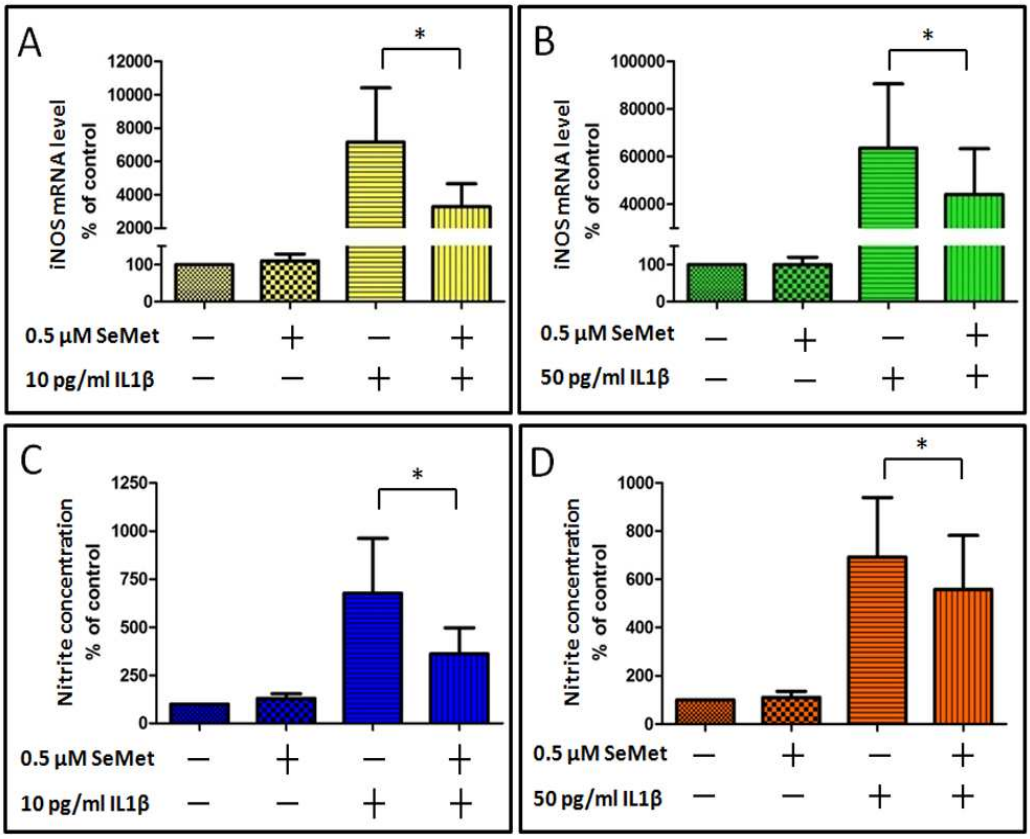


Figure 3.1 SeMet inhibits IL-1β-induced iNOS gene expression and NO production in primary human chondrocytes. Primary human chondrocytes were cultured for 24 hours in the absence (control) or presence of 0.5 µM SeMet, followed by 24 hour co-treatment without IL-1β (control) or with either 10 pg/ml (**A** and **C**) or 50 pg/ml (**B** and **D**) IL-1β. Gene expression for iNOS (**A** and **B**) was determined by RT-PCR normalized to 18S rRNA (average of triplicates for six independent experiments). Corresponding culture media were analyzed for nitrite concentration (**C** and **D**). Data were normalized to total DNA of the corresponding chondrocytes. Values shown are mean and SEM of iNOS or nitrite as a percentage of control (set at 100%). *P=0.031

3.3.2 Inhibitory Effect of SeMet on IL-1β-Induced COX2 Gene Expression and PEG_2 Production

Next we investigated if pretreatment of SeMet could also affect COX2 gene expression in the presence of IL-1β. Twenty four hour treatment with IL-1β at 10 pg/ml and 50 pg/ml significantly induced COX2 mRNA (approximately 53 fold and 120 fold respectively) compared to the control without IL-1β (Figures 3.2A and 3.2B). SeMet had no effect on the basal COX2 mRNA level. Twenty four hour pretreatment with SeMet significantly repressed IL-1β-induced COX2 steady state mRNA level by approximately 65% at 10 pg/ml IL-1β (p=0.031) and by approximately 50% at 50 pg/ml IL-1β (P=0.031). We further examined the effect of SeMet on PGE_2 production, the downstream enzymatic product of COX2. SeMet pretreatment inhibited IL-1β-induced PGE_2 production, by 32% at 10 pg/ml IL-1β (P=0.031), and by 24% at 50 pg/ml IL-1β (P=0.031) (Figures 3.2C and 3.2D).

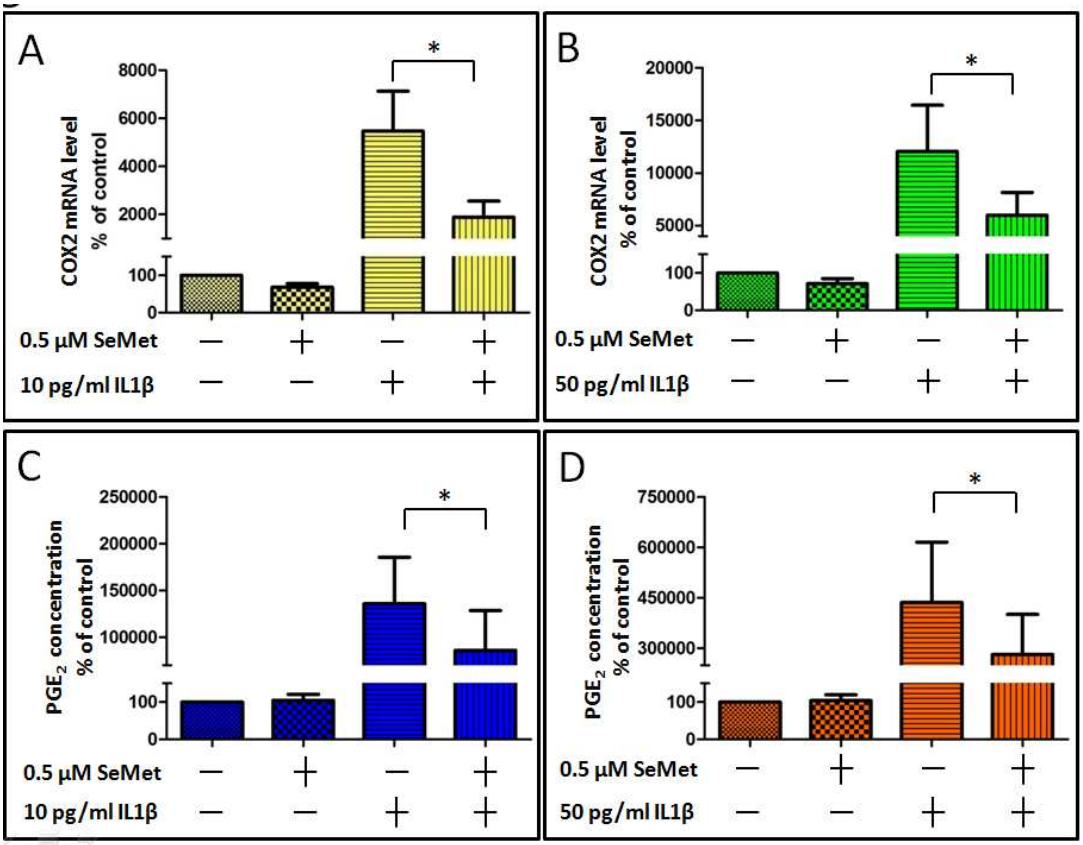


Figure 3.2 SeMet inhibits IL-1β-induced COX2 gene expression and PGE_2 production in primary human chondrocytes. Primary human chondrocytes were cultured for 24 hours in the absence (control) or presence of 0.5 µM SeMet, followed by 24 hour co-treatment without IL-1β (control) or with either 10 pg/ml (**A** and **C**) or 50 pg/ml (**B** and **D**) IL-1β. Gene expression for COX2 (**A** and **B**) was determined by RT-PCR normalized to 18S rRNA (average of triplicates for six independent experiments). Corresponding culture media were analyzed for PGE_2 concentration (**C** and **D**). Data were normalized to total DNA of the corresponding chondrocytes. Values shown are mean and SEM of COX2 or PGE_2 as a percentage of control (set at 100%). *P=0.031

3.3.3 Effect of SeMet on IL-1β-Induced Signaling Pathways

To determine whether the inhibitory effect of SeMet could be associated with alterations of IL-1β-induced signaling pathways, we examined the phosphorylation status of signaling proteins in IL-1β-stimulated primary chondrocytes in the presence and absence of SeMet. Stimulation of chondrocytes with IL-1β resulted in activation of MAPK kinases, p38 MAPK, ERK1/2 and JNK as well as NFkB pathway signaling molecules, IKKα/β and NFkB p65, with phosphorylation peaking approximately 30 minutes after stimulation. SeMet pretreatment alone did not affect the basal phosphorylation level of any of the signaling molecules at time zero (Figure 4A, lane 5 versus lane 1). In contrast, SeMet attenuated IL-1β-induced phosphorylation of p38 MAPK (Figure 3.3A, top row) but not the ERKs, JNK, IKKα/β or NFkB p65 (Figure 3.3A). The total basal level of p38 MAPK was unaffected by either IL-1β or SeMet. The mean phosphorylation level for each signaling molecule was determined by Image J analyses (normalized to the α-tubulin control) from three separate experiments (Figure 3.3B). SeMet pretreatment only modestly (39%) but significantly ($p=0.039$) reduced the IL-1β-induced activation of p38 MAPK based on the total area under the curve (from 5 to 45 minutes), compared to the IL-1β treated condition (Figure 3.3B). Pretreatment of SeMet did not affect the IL-1β activation of the other molecules (Figures 3.3B) nor total p38 MAPK.

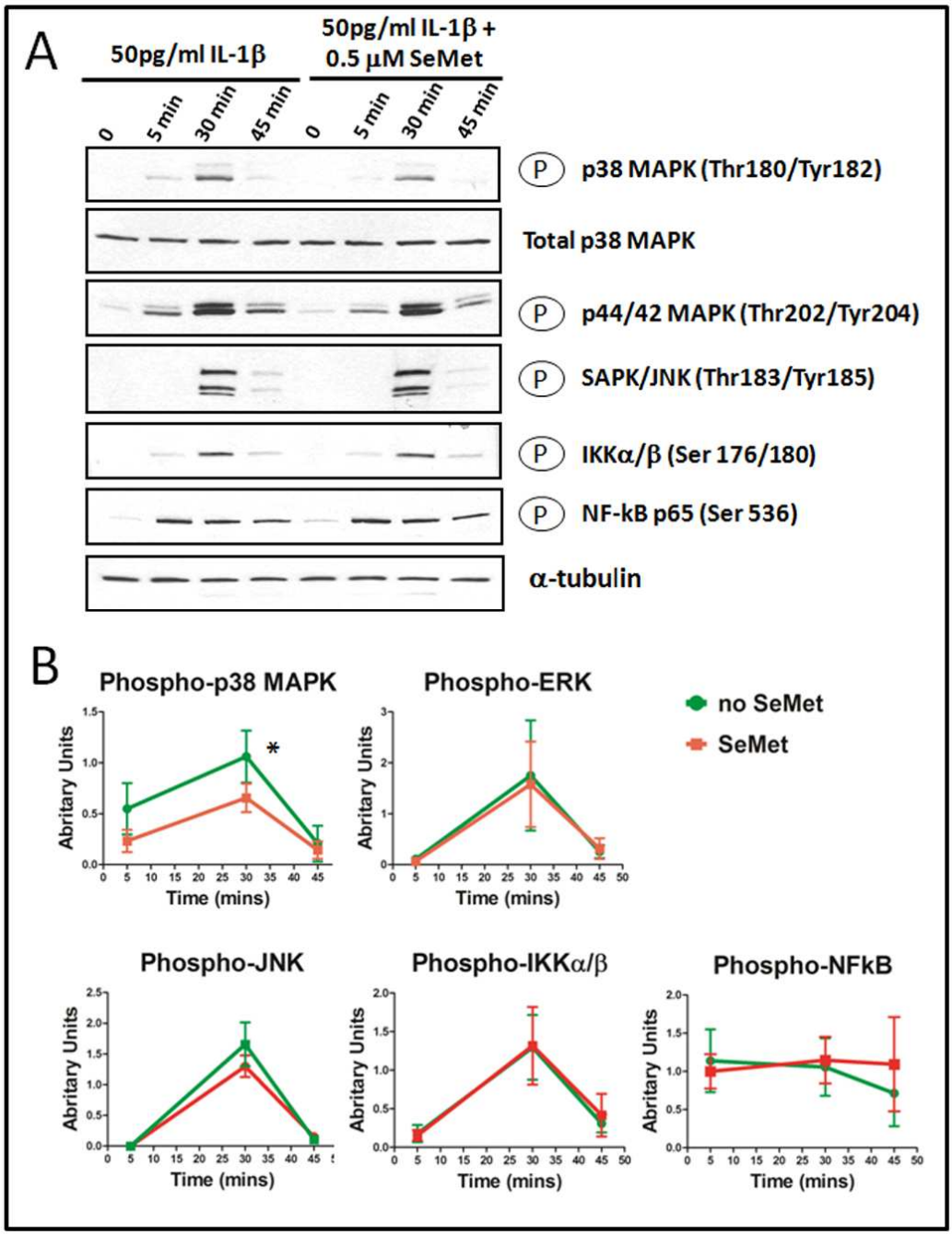


Figure 3.3 SeMet partially blocked IL-1β induced p38 MAPK (continued)

Figure 3.3 SeMet partially blocks IL-1β-induced p38 MAPK
(A) Western blot analysis. Primary human chondrocytes were cultured for 24 hours in the absence (control) or presence of 0.5 μM SeMet, and then co-treated with 50 pg/ml IL-1β treatment for 0, 5, 30 and 45 minutes. Equal amounts of total cell lysate were separated by SDS-PAGE and analyzed by Western blot for the following proteins: Phospho-p38 MAPK (Thr180/Tyr182), Total p38 MAPK, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Phospho-SAPK/JNK (Thr183/Tyr185), Phospho-IKKα/β (Ser176/180) and Phospho-NFkB p65 (Ser536); α tubulin (bottom row) was used as a control for normalization. The results shown are representative of three independent experiments.
(B) Quantification of the effects of SeMet on IL-1β-induced phosphorylation of signaling proteins. Phosphoprotein signaling intensity was quantified by Image J analysis of Western blots for up to 3 time points from three independent experiments (as described in A) with normalization to the internal control, α-tubulin. The normalized mean and SEM levels of phosphosignaling protein intensity are shown for Phospho-p38 MAPK (Thr180/Tyr182), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Phospho-SAPK/JNK (Thr183/Tyr185), Phospho-IKKα/β (Ser176/180) and Phospho-NFkB p65(Ser536). SeMet inhibited IL-1β activation of p38 MAPK significantly but not the generation of other phosphoproteins. *P=0.039.

3.4 Discussion

Elevated concentrations of the proinflammatory cytokine, IL-1, are found in the synovial fluid of OA joints (Goldring and Goldring 2004). IL-1 stimulates several key proinflammatory mediators such as PGE_2 and NO, which have been implicated in the pathogenesis of OA (Jacques, Gosset et al. 2006; Abramson 2008). IL-1 stimulates NO and PGE_2 through regulation of iNOS and COX2 gene expression respectively (Charles, Palmer et al. 1993; Geng, Blanco et al. 1995; Vuolteenaho, Moilanen et al. 2001). Our data demonstrated that pretreatment of chondrocytes with the antioxidant SeMet significantly inhibited production of both NO and PGE_2 in response to IL-1β. These results have potential clinical significance based on the known involvement of PGE_2 and NO in joint metabolism, as summarized below.

PGE_2 has protean manifestations - some catabolic and some anabolic. This is thought to be mediated by different EP receptors such as EP_2 and EP_4 (Attur, Al-Mussawir et al. 2008; Li, Ellman et al. 2009). PGE_2 upregulates matrix metalloproteinases (MMPs) which cause joint cartilage degradation (Tung, Arnold et al. 2002). PGE_2 has been shown to positively modulate type II collagen gene expression in cultured chondrocytes (Goldring, Suen et al. 1996). Furthermore, PGE_2 promotes differentiation and proliferation of growth plate chondrocytes (Schwartz, Gilley et al. 1998; Brochhausen, Neuland et al. 2006).

Sensitization of peripheral nociceptors by PGE_2 is believed to contribute to pain associated with inflammation (Dray and Read 2007).

NO, another key mediator downstream of IL-1, has been demonstrated to have an impact on cartilage homeostasis by regulating the balance of anabolic and catabolic metabolism. NO inhibits collagen and proteoglycan synthesis of cartilage *in vitro* (Taskiran, Stefanovic-Racic et al. 1994), and also induces matrix metalloproteinase synthesis in articular chondrocytes (Sasaki, Hattori et al. 1998). In addition, NO has been shown to reduce IL-1 receptor antagonist (IL-1ra) synthesis by chondrocytes (Pelletier, Mineau et al. 1996). Incubation of human articular chondrocytes with the NO donor, sodium nitroprusside (SNP), activates apoptotic gene expression (Maneiro, Lopez-Armada et al. 2005). NO can trigger apoptosis by a mitochondria-dependent mechanism that could be mediated through regulation of expression of apoptotic-related genes such as caspase- 3 and 7 (Maneiro, Lopez-Armada et al. 2005; Wu, Chen et al. 2007). Furthermore, 3-nitrotyrosine, a stable product formed by reaction of NO and reactive oxygen species, has correlated with IL-1β-induced oxidative stress in aging and osteoarthritic cartilage tissue (Loeser, Carlson et al. 2002).

The selenium-mediated reduction in the NO and PGE_2 production was associated with corresponding reductions in iNOS and COX2 transcript levels. The direction and magnitude of the effect of SeMet on the IL-1β-induced NO production was similar to the effect on iNOS mRNA expression. Therefore, the

full effect of SeMet to modulate NO production is likely mediated at a transcriptional level. The inhibitory effect of SeMet on IL-1β-induced NO production found in our study was similar to the inhibitory effect shown previously by others for the inorganic form of selenium, namely selenite (5-10μM), on LPS-induced iNOS gene expression and NO production in murine macrophages (Kim, Johnson et al. 2004; Yun, Yang et al. 2007).

In the case of PGE_2 production, the magnitude of the inhibition of PGE_2 protein production was approximately 50% less than the magnitude of inhibition of COX2 gene expression. It is known that inhibition of NO may lead to a corresponding compensatory release in PGE_2 (Fermor, Weinberg et al. 2005). Therefore, we believe the PGE_2 results represent a combination of downstream effects: SeMet inhibition of COX2 gene expression; and SeMet inhibition of NO leading to increase in PGE_2. These NO and PGE_2 data provide a plausible mechanism whereby Se may play a protective role in OA.

Signaling pathways induced by IL-1β, that regulate NO and PGE_2 production, are sensitive to the redox state of cells, and can be altered by natural antioxidants such as curcumin and capparis spinosa (Panico, Cardile et al. 2005; Shakibaei, John et al. 2007). Cytokines, including IL-1, have been shown to induce NO and PGE_2 production through MAPK and NFkB pathways in human and rat chondrocytes (Thomas, Thirion et al. 2002; Masuko-Hongo, Berenbaum et al. 2004; Lianxu, Hongti et al. 2006). We demonstrated that selenium partially

inhibited the IL-1β-induced phosphorylation of p38 MAPK, but not other MAPKs that are commonly involved in IL-1β cell signaling. This is consistent with a past study that also showed selective targeting of p38 MAPK by selenite, an inorganic form of selenium (Kim, Johnson et al. 2004). However, we cannot exclude the fact that selenium may alter IL-1β-induced iNOS or COX2 gene expression through other signaling pathways since our study was limited by having investigated only a single form of selenium, SeMet. Selenate (another inorganic form of Se) has been shown to activate the PI3K/AKT pathway (Heart and Sung 2003), which negatively regulates LPS-induced COX2 (Monick, Robeff et al. 2002). In contrast to our results, generated with human chondrocytes and physiologically relevant concentrations of SeMet and IL-1β (10 and 50 pg/ml), a previous study utilizing bovine chondrocytes and supra-physiological doses of IL-1β (10 ng/ml), failed to observe an effect of SeMet on IL-1β-induced p38 kinase activity (Andriamanalijaona, Kypriotou et al. 2005). Thus, the results are likely dependent on the dose and form of selenium used. By the same token, the dose and form of selenium we chose may also explain why SeMet did not have a significant effect on phosphorylation of NFkB p65 while high doses of selenite have been shown to inhibit NFkB activity in cells other than chondrocytes, such as macrophages (Zamamiri-Davis, Lu et al. 2002; Kim, Johnson et al. 2004; Yun, Yang et al. 2007). Finally, in contrast to other studies that measured the DNA

binding activities of NFkB, we examined the phosphorylation level of NFkB p65 which might account for different results between studies.

The mechanism whereby selenium modifies the phosphorylation state of p38 MAPK is unknown. Selenium may regulate the redox state which may alter the activity of upstream kinases or phosphatases that in turn control the phosphorylation status of p38 MAPK and hence the downstream signaling. Sulfhydryl groups of protein tyrosine phosphatase can be reversibly oxidized and the enzymatic activity regulated (Monteiro, Ivaschenko et al. 1991). By analogy, regulation of the redox state by the antioxidant selenium might explain the selenium-related modulation of IL-1β signaling. Further work remains to be done to elucidate the mechanisms of selenium-mediated regulation of IL-1β signaling.

In conclusion, we have shown that the antioxidant SeMet inhibits IL-1β-induced NO and PGE_2 production through modulation of iNOS and COX2 gene expression in primary chondrocytes. Our study also shows that selenium partially blocked IL-1 activation of the p38 MAPK pathway. These results suggest that one mechanism whereby selenium may exert a protective effect is through regulation of the expression of inflammation related genes, possibly mediated in part through inhibition of IL-1β cell signaling.

CHAPTER 4

Selenoproteins Modify the Response of Chondrocytes to Inflammatory Stimuli

4.1 Introduction

Several lines of evidence suggest that selenium may have a protective role in osteoarthritis (OA). Profound selenium deficiency is associated with the severe osteoarthropathy known as Kashin-Beck Disease (KBD) that affects around 7 million individuals in China and neighboring regions (Allander 1994). In the US, a low but non-deficiency level of selenium has been shown to be associated with OA presence and severity in a large population study (Jordan, Fang et al. 2005; Jordan, Fang et al. 2007). These observations suggest a requirement for selenium for cartilage health and OA prevention. Recently, selenium has been shown to be anti-inflammatory by altering iNOS and COX2 gene expression in response to lipopolysaccharide (LPS) stimulation in cultured macrophages (Prabhu, Zamamiri-Davis et al. 2002; Vunta, Belda et al. 2008). Our recent study has demonstrated that pretreatment of chondrocytes with selenomethionine (SeMet) attenuates IL-1β induced NO and PGE_2 production (Cheng, Stabler et al. 2010). Given the fact that selenium can function through selenoproteins, we hypothesize that specific selenoproteins mediate the antioxidative and anti-inflammatory effect of selenium in chondrocytes

To date, twenty five mammalian selenoproteins have been identified including three large subfamilies: glutathione peroxidases (GPXs), thioredoxin reductases (TRs), and iodothyronine deiodinases (DIOs) (Kryukov, Castellano et al. 2003). GPXs and TRs, antioxidative enzymes, catalyze the reduction of

intracellular peroxide and regulate the redox balance in cells (Burk 1990). Several studies now implicate specific selenoproteins in OA. A genetic variant of GPX1 and reduced plasma GPX activity is associated with a risk of developing KBD (Xiong, Mo et al. 2010). Gene expression of GPX1 and thioredoxin-interacting protein (TXNIP) is downregulated in cartilage lesions with moderate to severe late-stage osteoarthritis (Aigner, Fundel et al. 2006). The DIOs regulate the bioactivity of thyroid hormone by controlling levels of triiodothyronine (T3) and thyroxine (T4) (Bianco, Salvatore et al. 2002). A recent genetic study showed that a variant of DIO2, responsible for local (chondrocyte) production of T3 from T4, was associated with risk for developing OA (Meulenbelt, Min et al. 2008). However, the exact role of DIO2 in the pathogenesis of OA remains to be determined. In a bacterial infected mouse model, DIO2 mRNA expression was inversely correlated with IL-1β expression in skeletal muscle (Kwakkel, van Beeren et al. 2009). Taken together, this evidence suggests roles for GPX1, thioredoxins, and DIO2 in OA susceptibility and inflammatory responses. Our goal in this study was to elucidate the role of specific major selenoproteins, GPX1, TR1 and DIO2, in the pathogenesis of OA through analysis of inflammatory responses in chondrocytes when these selenoproteins were depleted by RNA interference.

4.2 Materials and Method

4.2.1 Chondrocyte Isolation and Culture

Anonymous surgical waste articular cartilage samples, taken at the time of joint replacement, were used for this project. Tissue was collected under the approval of the Duke IRB who determined that this protocol met the definition of research not involving human subjects as described in 45CFR46.102(f) and satisfied the Privacy Rule as described in 45CFR164.514. Articular cartilage samples were obtained from 6 patients undergoing total knee replacement surgery [mean age, 60.3 +/- 10.3 years]. Cartilage was harvested from nonlesional areas, further minced, and subjected to pronase and collagenase digestion to isolate primary chondrocytes, similar to previously published methods (Kuettner, Pauli et al. 1982). Isolated chondrocytes within the first two passages were used for all experiments.

4.2.2 RNA interference (RNAi) of GPX1, TR1 and DIO2

RNAi transfection was performed using the program U20 of the Amaxa Nucleofector (Gaithersburg, MD), with either the Amaxa Primary Human Chondrocyte Nucleofector Kit or Mirus Ingenio^{TM} Electroporation Kit (Madison, MD) according to the manufacturer's protocols. Chondrocytes were transfected with the following siRNAs: 3 µg of Silencer Negative Control No.1 siRNA

(Ambion, Applied Biosystem) served as a scrambled transfection control: 1 µg of human GPX1 specific siRNA (s804); 3 µg of human TR1 specific siRNA (s755); or 3 µg of human DIO2 specific siRNA (s4106). After transfection, cells were cultured for 48 hours in DMEM/F12 supplemented with 10% fetal bovine serum to allow gene suppression and turnover of the preexisting targeted proteins. Transfected cells were subsequently serum-starved in DMEM media with or without 0.5 µM SeMet treatment for 24 hours, then treated for another 24 hours with 10 pg/ml IL-1β (R & D systems, Minneapolis, MN) with or without 0.5 µM SeMet. The IL-1β concentration of 10 pg/ml and the SeMet concentration of 0.5 µM were chosen based on evidence for physiological relevance (equivalent to concentrations in human OA synovial fluid) and previously established experiments (Westacott, Whicher et al. 1990; Kahle, Saal et al. 1992; Yazar, Sarban et al. 2005; Cheng, Stabler et al. 2010). Cells were treated with RNeasy Lysis buffer (Qiagen Valencia, CA) to isolate RNA for gene expression studies, or with lysis buffer (10 mM HEPES, pH 7.5, 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA and 1 M NaCl) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO) to produce total protein cell lysates for Western blot analysis.

4.2.3 RNA Isolation and Real Time RT-PCR

Cell lysates, prepared by RNeasy Lysis buffer from each experimental condition were homogenized by passage through a QIAshredder spin column

(Qiagen, Valencia, CA). The total DNA and RNA fractions were further isolated using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The isolated total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) for Real Time RT-PCR analysis. The ABI Prism 7000 sequence detection system and relative quantification software (Applied Biosystems, Foster City, CA) were used for real-time analyses. The amplification for real-time RT-PCR used the following Applied Biosystems primer and probe sets: 18S rRNA endogenous control; Hs00829989_gH (GPX1); Hs00182418_ml (TR1); Hs00255341_ml (DIO2); Hs01573474_g1(COX2); and Hs01555410_m1 (IL-1β) The real-time reactions were performed in triplicate in a final volume of 25 µl.

Raw mRNA expression values were computed by $2^{-\Delta Ct}$ formula (Livak and Schmittgen 2001) with values normalized to 18S rRNA, where ΔCt represents the difference in Ct (threshold cycle) number of the 18S rRNA gene and the COX2 or IL-1β genes. Results were derived from a total of 4 independent experiments for COX2 and IL-1β gene expression, performed in triplicate, using a total of 4 separate primary chondrocyte cell lines. The relative fold changes in mRNA expression levels of COX2 and IL-1β were calculated by the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen 2001), between cells transfected with selenoprotein siRNA and the cells transfected with scrambled siRNA in different

treatments. Paired t-test comparing subgroups were performed based on the log transformed $2^{-\Delta Ct}$ values. P values less than 0.05 were considered significant after correction for multiple comparisons (Bonferroni).

4.2.4 Western Blot Analysis

Whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. Membranes were blocked with 5% BSA in TBS/0.1% Tween 20 (TBS-T). Polyclonal primary antibodies against GPX1, TR1 and DIO2 were obtained from Abcam (Cambridge, MA). A monoclonal antibody against α-tubulin (Sigma) was used as a normalization control at 1:10,000 dilution. Anti-rabbit and anti-mouse IgG-HRP (Jackson ImmunoResearch, West Grove, PA) secondary antibodies were used at a 1:5,000 dilution. The resulting films were scanned using CanoScan LiDE 70 (Canon, Lake Success, NY) and the band intensities were quantified using Adobe Photoshop CS and Image J (National Institutes of Health, Bethesda, MD).

4.3 Results

4.3.1 Suppression of Selenoproteins by RNAi

To examine the role of GPX1, TR1 and DIO2 in mitigating the IL-1β response in primary human chondrocytes, we suppressed the expression of GPX1, TR1, and DIO2 with sequence specific siRNA (Figure 4.1). Compared with the scrambled siRNA, GPX1 mRNA levels were significantly decreased 96% by GPX1 siRNA ($P<0.0001$) (Figure 4.1A). TR1 mRNA expression was significantly decreased 66% by TR1 siRNA ($P<0.0001$), and DIO2 mRNA expression was significantly decreased 51% by DIO2 siRNA ($P<0.01$). Compared with the protein concentrations in cells transfected with the scrambled siRNA, protein expression of each of the targeted selenoproteins was also reduced (Figure 4.1B): GPX1 was reduced by approximately 80%; TR1 was reduced by approximately 80%; and DIO2 was reduced by approximately 50%.

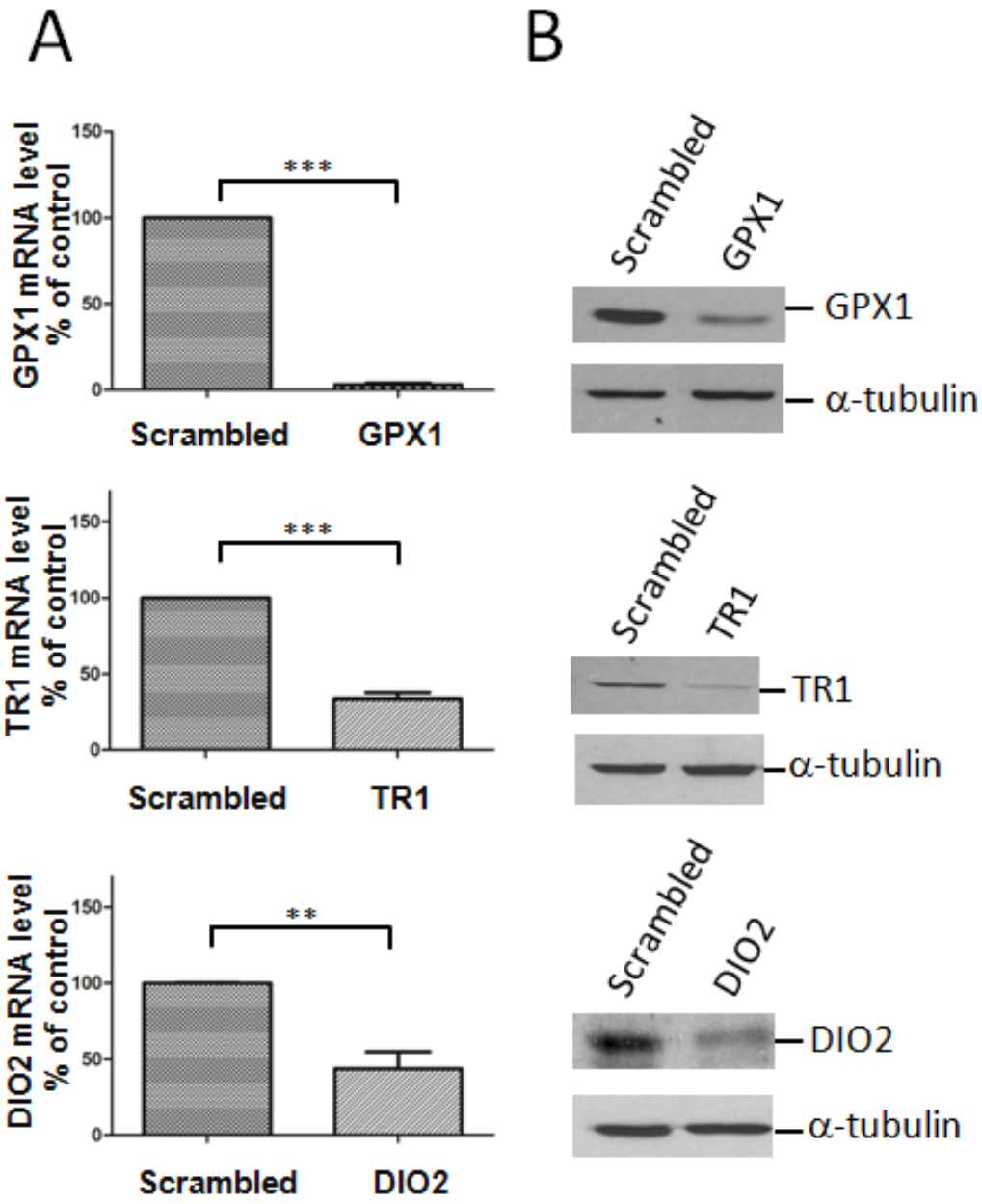


Figure 4.1 Suppression of glutathione peroxidase 1 (GPX1), thioredoxin reductase 1 (TR1) and iodothyronine deiodinase 2 (DIO2) (continued)

Figure 4.1 Suppression of glutathione peroxidase 1 (GPX1), thioredoxin reductase 1 (TR1) and iodothyronine deiodinase 2 (DIO2)
Primary human chondrocytes were transfected with a scrambled control small interfering RNA (siRNA) or siRNA specific for GPX1, TR1 and DIO2. GPX1, TR1 and DIO2 stead-state mRNA levels and proteins levels were examined in the transfected cells in 72-96 hours post-transfection. **A,** GPX1, TR1 and DIO2 mRNA levels, were determined by RT-PCR normalized to18S rRNA in siRNA-transfected cells (average of triplicates for four independent experiments). Values are the mean and SEM levels of mRNA for GPX1, TR1 or DIO2 expressed as a percentage of the scrambled control. The mRNA level in cells transfected with the scrambled siRNA was set at 100%. *** $P<0.001$ or ** $P<0.01$ versus scrambled control (scrambled) **B.** GPX1, TR1 and DIO2 protein expression, were determined by Western blotting, in siRNA-transfected cells. Equal amounts of total cell lysate were separated by SDS-PAGE and analyzed by Western blot, α-tubulin (bottom row) was used as a control for normalization.

4.3.2 COX2 Gene Expression in the Setting of Suppressed Selenoprotein Expression

The suppression of DIO2 resulted in an increase in COX2 basal mRNA expression, both in the presence ($p=0.0005$) and absence ($P<0.001$) of selenium (Figure 4.2). The suppression of DIO2 also resulted in an increase in IL-1β-induced COX2 steady state mRNA expression in the absence ($P<0.001$) of selenium, and blocked the ability of selenium to downregulate IL-1β-induced COX2 steady state mRNA expression ($p<0.001$). In contrast, the suppression of GPX1 and TR1 had no significant effect on basal level expression of COX2. However, the suppression of TR1 blocked the ability of selenium to downregulate IL-1β-induced COX2 mRNA expression.

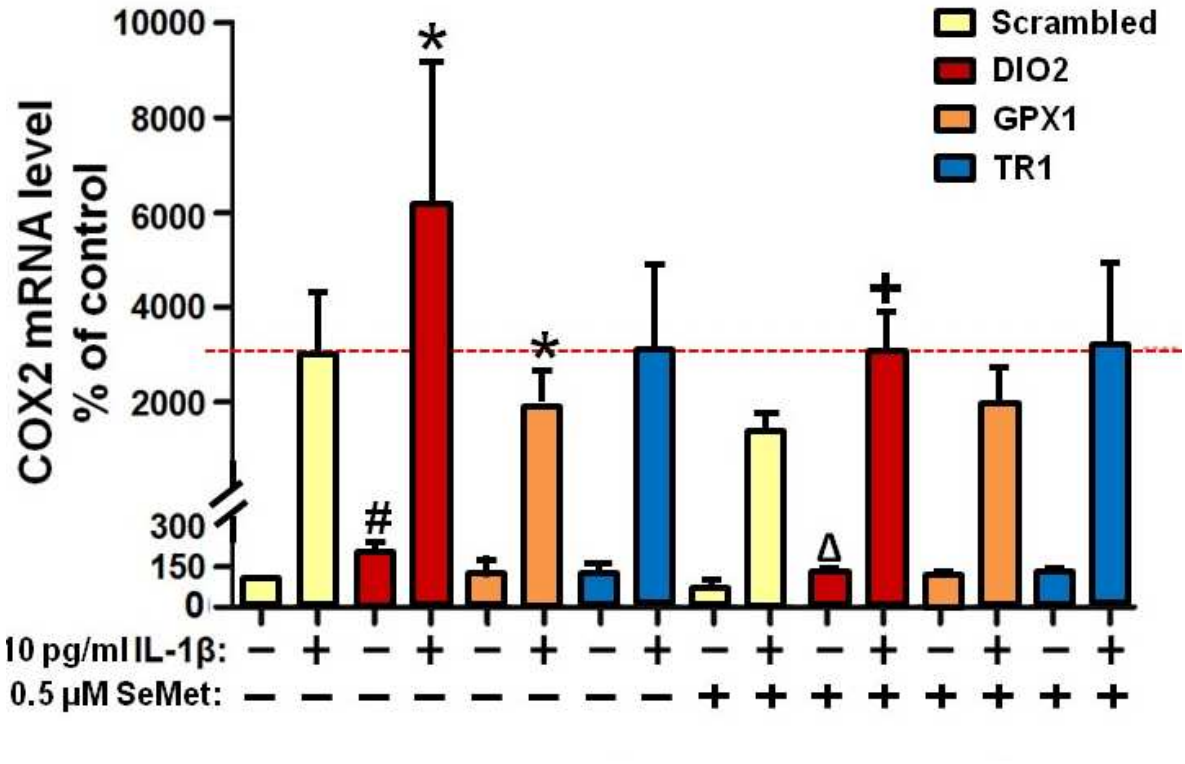


Figure 4.2 Effect of depleting selenoproteins on IL-1β-induced COX2 gene expression in primary chondrocytes (continued)

Figure 4.2 Effect of depleting selenoproteins on IL-1β-induced COX2 gene expression in primary chondrocytes

Primary human chondrocytes were transfected with a scrambled control small interfering RNA (siRNA) or siRNA specific for GPX1, TR1 and DIO2. After 48 hours post-transfection, siRNA transfected cells were cultured for 24 hours in the absence (Se deficient) or presence of 0.5 µM SeMet (Se adequate), followed by 24 hour co-treatment without IL-1β or with 10 pg/ml IL-β. Gene expression for COX2 was determined by RT-PCR normalized to 18S rRNA (average of triplicates for four independent experiments). The effect of depleting individual selenoproteins on IL-1β-induced COX2 gene expression is shown under Se deficient and Se adequate conditions. Values shown are mean and SEM of COX2 as a percentage of scrambled control without neither IL-1β nor SeMet treatment (set at 100%) from four independent experiments. # $P<0.001$ versus scrambled without SeMet and IL-1β; *$P<0.001$ versus scrambled with IL-1β stimulation in the absence of SeMet; Δ $P<0.001$ versus scrambled without IL-1β stimulation in the presence of SeMet; and + $P<0.05$ versus scrambled with IL-1β stimulation in the presence of SeMet.

4.3.3 IL-1β Gene Expression in the Setting of Suppressed Selenoprotein Expression.

In order to elucidate if the effect of depleting selenoprotein proteins on IL-1β-induced COX2 gene expression was due to the change in IL-1β mRNA levels, we examined the mRNA level of IL-1β in selenoprotein-depleted conditions. Similar to the effect on COX2 gene expression, the suppression of DIO2 resulted in an increase in IL-1β basal steady state expression (Figure 4.3). The suppression of DIO2 also resulted in an increase in IL-1β-induced IL-1β steady state mRNA expression in the absence of selenium ($p<0.01$), and blocked the ability of selenium to downregulated IL-1β induced IL-1β steady state mRNA expression ($p<0.001$). However, the suppression of GPX1 and TR1 had no significant effect on basal level expression of IL-1β in the absence of selenium. The suppression of TR1 blocked the ability of selenium to downregulate IL-1β-induced IL-1β mRNA expression ($p<0.001$).

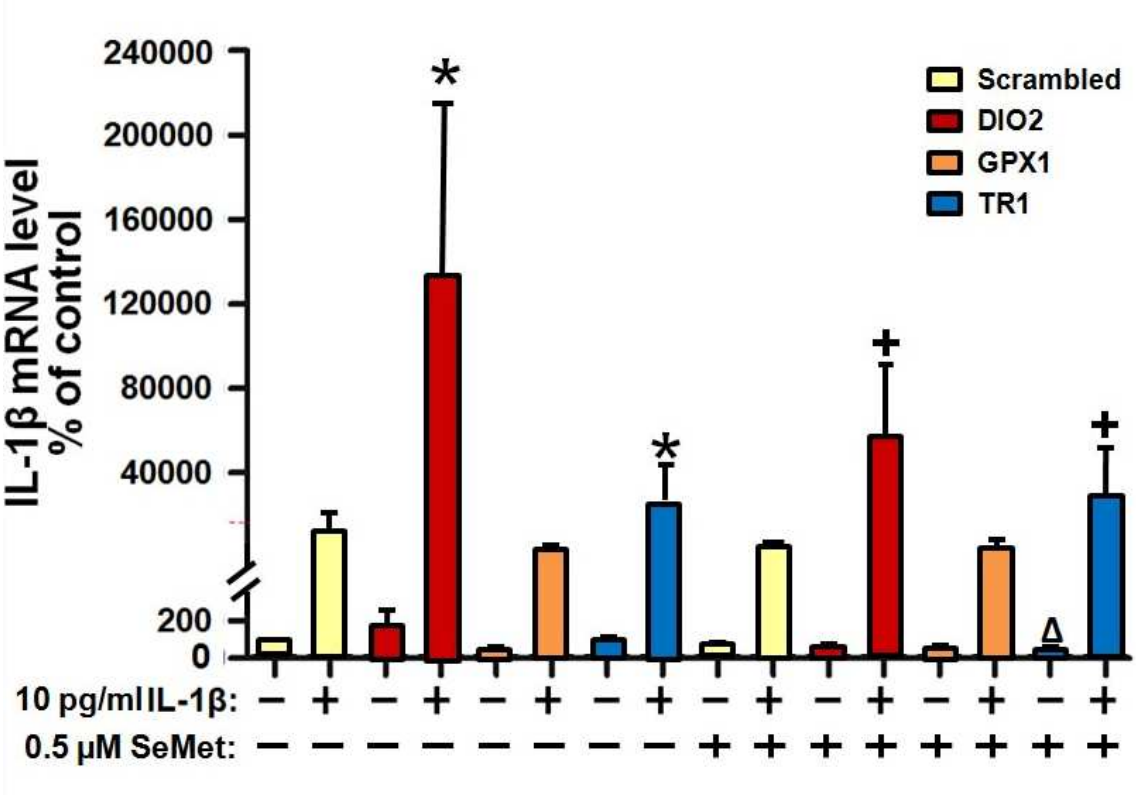


Figure 4.3 Effect of depleting selenoproteins on IL-1β-induced IL-1β gene expression in primary chondrocytes (continued)

Figure 4.3 Effect of depleting selenoproteins on IL-1β-induced IL-1β gene expression in primary chondrocytes

Primary human chondrocytes were transfected with a scrambled control small interfering RNA (siRNA) or siRNA specific for GPX1, TR1 and DIO2. After 48 hours post-transfection, siRNA transfected cells were cultured for 24 hours in the absence (Se deficient) or presence of 0.5 µM SeMet (Se adequate), followed by 24 hour co-treatment without IL-1β or with 10 pg/ml IL-β. Gene expression for IL-1β was determined by RT-PCR normalized to 18S rRNA (average of triplicates for four independent experiments). The effect of depleting individual selenoproteins on IL-1β-induced IL-1β gene expression is shown under Se deficient and Se adequate conditions. Values shown are mean and SEM of IL-1β as a percentage of scrambled control without neither IL-1β nor SeMet treatment (set at 100%) from four independent experiments. * $P<0.05$ versus scrambled without SeMet and IL-1β; Δ $P<0.001$ versus scrambled without IL-1β stimulation in the presence of SeMet; and + $P<0.001$ versus scrambled with IL-1β stimulation in the presence of SeMet

4.4 Discussion

Selenium is an essential trace element involved in several key metabolic activities such as protection against oxidative damage and regulation of thyroid function (Rayman 2000; Reilly 2006). Selenium is distinct from other antioxidants by incorporating itself as a selenocysteine at the key catalytic site of selenoproteins. In chondrocytes, depletion of DIO2 and TR1 blocked the ability of selenium to downregulate IL-1β-induced COX2 gene expression. These results can be interpreted from the increase of IL-1β mRNA level itself in DIO2- and TR1- depleted conditions, (Figure 3) which may oppose the inhibitory effect of selenium on IL-1β. Furthermore, evidence showed that thioredoxin, a substrate of TR1, modulated inflammatory responses in asthma and hepatic fibrosis animal models (Ichiki, Hoshino et al. 2005; Okuyama, Nakamura et al. 2005) . Our data showed that depletion of DIO2 increased both basal and IL-1β-induced IL-1β gene expression. This is in consistent with a previous study that demonstrated that DIO2 level is inversely correlated with IL-1β in skeletal muscle (Kwakkel, van Beeren et al. 2009). This suggests that DIO2 or T3 may negatively modulate the IL-1β response by regulating IL-1β gene expression. Given that T3 is the enzymatic product of DIO2, the data also suggest that T3 may have an anti-inflammatory effect (as previously reported) and the anti-inflammatory effect of T3 may promote repair of the conditions of chronic demyelination (Harsan, Steibel et al. 2008). A recent study demonstrated that activation of Liver X

Receptor (LXR) decreased basal and IL-β- induced PGE_2 production in human cartilage explants and relieved joint pain in a rat OA model (Li, Rivera-Bermudez et al. 2010). Expression of LXR α has been shown to be positively regulated by thyroid hormone in HepG2 cells (Hashimoto, Matsumoto et al. 2007). These studies suggest that thyroid hormone T3, produced by DIO2, may suppress PGE_2 production indirectly through activation of LXR. This interpretation is aligned with the reasoning in our study that activation or maintenance of LXR activity by thyroid hormone may be one of the mechanisms by which DIO2 suppresses IL-1β-induced COX2 expression in addition to modulating IL-1β mRNA levels.

Our study highlights a potential new role of DIO2 in modulating the inflammatory response in chondrocytes. Further study to elucidate the mechanism by which DIO2 or T3 modulates the inflammatory response would be beneficial in determining the role of DIO2 in the pathogenesis of OA. While depleting DIO2 may increase cytokine induced inflammatory response shown in our study, DIO2 expression was highly upregulated in late stage OA cartilage (Ijiri, Zerbini et al. 2008). However, whether the overexpression of DIO2 contributes to the pathogenesis of OA is unclear. It is possible that upregulation of DIO2 in OA cartilage may be a counter-measure based on the fact that DIO2 has a protective role to oppose the inflammatory response in chondrocytes. This would suggest that optimal DIO2 activity is essential for maintenance of normal

chondrocyte health in cartilage through production of adequate local T3 concentrations. It is interesting that profound depletion of GPX1 by (both mRNA and protein) did not have a significant impact on COX2 or IL-1β gene expression. Depleting GPX1 would be expected to have a similar impact compared to TR1 based on the fact that they both are antioxidative enzymes. Although we focused on the effects of depleting GPX1, TR1 and DIO2, we cannot exclude the possibility that depletion of these selenoproteins may impact the expression of other selenoproteins. Profound depletion of GPX1 may trigger gene expression of other antioxidative enzyme to compensate for its reduced function.

In conclusion, we have demonstrated that depletion of DIO2 increased IL-1β-induced COX2 gene expression in chondrocytes and blocked the ability of selenium rescue*, i.e.* selenium's ability to downregulate the IL-1β-induced COX2 expression. Similarly, TR1 depletion blocked the ability of selenium to downregulate the IL-1β-induced COX2 expression. We also showed that depleting DIO2 increased IL-1β-induced IL-1β gene expression which suggests DIO2 may negatively modulate the IL-1β response through regulation of IL-1β gene expression itself. These data suggest that selenium may exert its protective effect through antioxidative and thyroid hormone-activating selenoproteins in suppressing the IL-1β induced inflammatory response in chondrocytes.

CHAPTER 5

Conclusions

5.1 Thesis Conclusions

In this project, we investigated the chondroprotective mechanisms of selenium in chondrocytes. We hypothesized that selenium exerts its chondroprotective benefit via an anti-oxidative and anti-inflammatory effect, mediated by specific selenoproteins that neutralize cytokine-induced inflammatory responses. In order to test this hypothesis, our aims were three-fold. First, we established a model system for evaluating the effect of selenium in human chondrocytes. Second, we examined the effect of selenium on inflammatory genes in human chondrocytes. Finally, we evaluated the role of major selenoproteins as mediators of the anti-inflammatory effect of selenium in human chondrocytes.

We compared the toxicity of selenite and selenomethionine (SeMet) in SW-1353 cell lines. SeMet has been shown to be less toxic compared to selenite. We subsequently chose a concentration of 0.5 µM for SeMet in this study as it is physiologically relevant. We determined the optimal time for serum starvation in order to deplete the cellular selenium storage under the conditions that we used for cell culture. We demonstrated that 24 to 48 hours of serum starvation led to a dramatic decrease in protein levels of GPX1 and GPX4, markers of cellular selenium status, without significant impact on cell viability. SeMet increased GPX1 and 4 transcript levels, GPX1 and 4 protein levels, as well as cellular glutathione peroxidase activity in SW-1353 cells. Similarly, SeMet

increased TR1 protein levels and cellular thioredoxin reductase activity in SW-1353 cells. These findings demonstrate the ability of selenium to induce the expression and activity of antioxidative enzymes, which could thereafter neutralize excessive reactive oxygen species generated from various stimuli. We also defined a concentration range of IL-1β to be 10-100 pg/ml based on the dose-response analysis of IL-1β stimulated MMP expressions; this concentration range is physiologically relevant. Finally, we demonstrated that selenium inhibited IL-1β-induced MMP1 and MMP13 gene expression and total MMP activities in primary chondrocytes in our settings. Therefore, we have established a working system for studying the effect of selenium in chondrocytes. Furthermore, our data suggest selenium may exert its protective effect by regulating antioxidative enzyme expression and neutralizing the catabolic effect of IL-1β.

We examined the effect of SeMet on IL-1β-induced NO and PGE_2 production as well as iNOS and COX2 gene expressions. SeMet inhibited IL-1β induced production of both NO and PGE_2 in primary chondrocytes. This was associated with a decrease in iNOS and COX2 transcript levels. In order to determine whether the inhibitory effect of SeMet could be associated with common IL-1β-induced signaling pathways, we examined the activation profiles of p38 MAPK, ERK1/2, JNK and NFkB pathways stimulated by IL-1β in the absence and presence of SeMet. SeMet partially blocked IL-1β-induced

phosphorylation of p38 MAPK but not the ERKs, JNK, IKKα/β or NFkB p65. These findings suggest another protective mechanism whereby selenium regulates the expression of inflammation-related genes, possibly mediated through inhibition of IL-1β cell signaling.

In order to elucidate the role of three specific selenoproteins GPX1, TR1 and DIO2 in mediating the inflammatory response, we measured IL-1β induced COX2 and IL-1β transcript levels when these selenoproteins were depleted by RNA interference. We showed that depletion of DIO2 increased IL-1β induced COX2 gene expression in chondrocytes and blocked the ability of selenium to downregulate IL-1β-induced COX2 expression. Similarly, TR1 depletion blocked the ability of selenium to inhibit IL-1β-induced COX2 expression. The increased IL-1β-induced COX2 gene expression was associated with elevated IL-1β expression in DIO2-depleted chondrocytes. Therefore, DIO2 may modulate the IL-1β response through regulating IL-1β gene expression itself. Our study highlighted a potential new role of DIO2 in modulating the inflammatory response in chondrocytes. These data suggest that selenium may exert its protective effect through antioxidative and thyroid hormone-activating selenoproteins in suppressing the IL-1β-induced inflammatory response in chondrocytes.

5.2 Potential Chondroprotective Mechanisms of Selenium

Overproduction of ROS in chondrocytes in response to different stimuli such as mechanical stress, cytokine stimulation and aging (Stadler, Stefanovic-Racic et al. 1991; Yamazaki, Fukuda et al. 2003; Loeser 2009), has been proposed as one mechanism in the pathogenesis of OA. Our data demonstrated that selenium induced the expression and activity of two antioxidative enzymes, GPXs and TR1, in SW-1353 cells. This suggests that selenium may exert its protective effect by enhancing antioxidative enzyme activity to neutralize overproduction of reactive oxygen species and hence the oxidative stress-related responses in chondrocytes (Figure 5.1).

On the other hand, we demonstrated that selenium inhibited IL-1β-induced NO and PGE_2 production as well as MMP activities and these were associated with corresponding reductions in iNOS, COX2, MMP1 and MMP13 transcript levels. We further showed that selenium partially inhibited the IL-1β-induced phosphorylation of p38 MAPK that is commonly involved in IL-1β cell signaling. Based on the key role of IL-1β and its downstream mediators in the pathogenesis of OA (Jacques, Gosset et al. 2006; Goldring and Marcu 2009), our data suggest that another protective mechanism of selenium may be through regulation of the inflammatory responses and catabolic effects of cytokines (Figure 5.2).

In elucidating the role of selenoproteins in mediating the inflammatory response, we have shown that depletion of DIO2 and TR1 blocked the inhibitory

effect of selenium on IL-1β-induced COX2 gene expression. Depletion of DIO2 also increased IL-1β-induced COX2 gene expression. This suggests that DIO2 or T3 (the enzymatic product of DIO2) may negatively modulate the IL-1β response. Our data also suggest that part of this inhibitory effect of DIO2 could be through regulation of IL-1β gene expression itself. These highlight the possibility that selenium exerts its effect through specific selenoproteins such as TR1 and DIO2 in modifying the IL-1β-induced inflammatory response in chondrocytes (Figure 5.3).

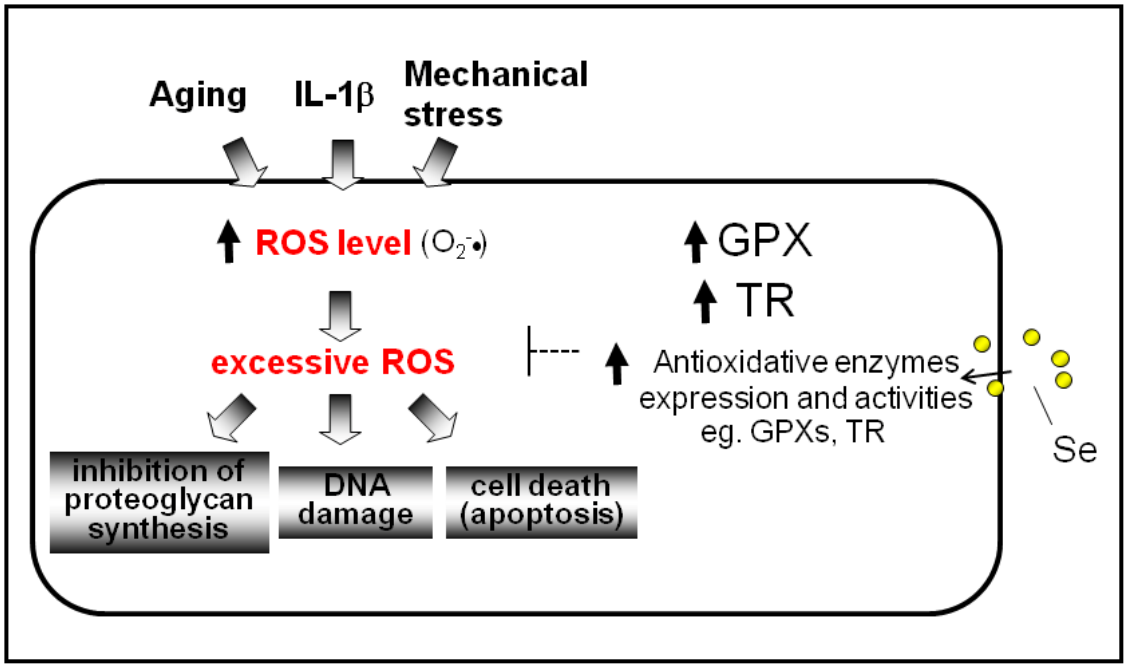


Figure 5.1 Selenium (Se) induces gene expression and activity of antioxidative enzymes, and neutralizes the overproduction of reactive oxygen species induced by various stimuli. Our data showed that selenium induced glutathione peroxidase (GPX) and thioredoxin reductase (TR) gene expression and corresponding enzyme activity in SW-1353 cells.

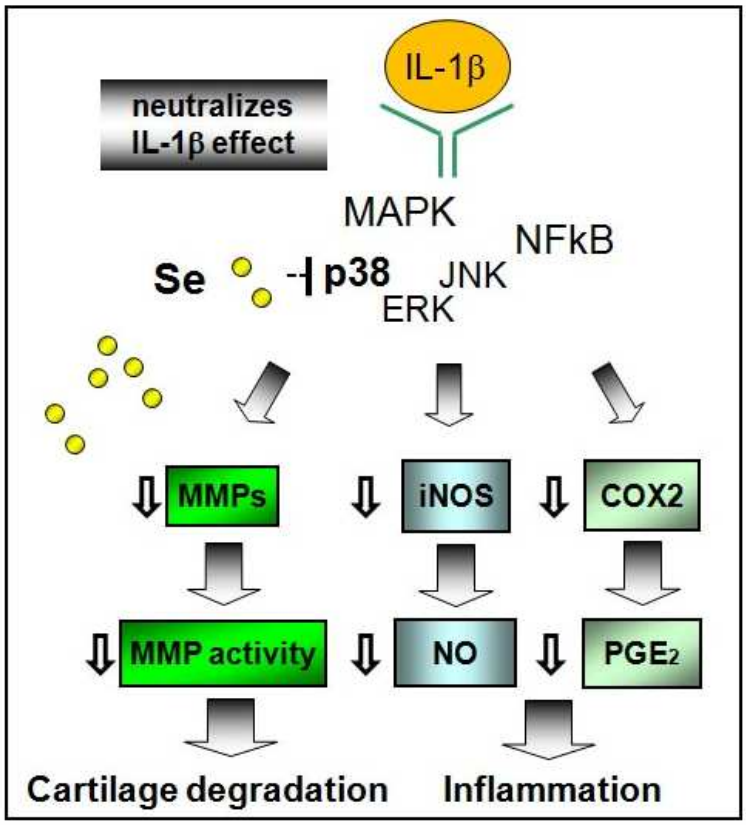


Figure 5.2 Selenium (Se) neutralizes the interleukin-1 beta (IL-1β)-induced inflammatory response in chondrocytes. Selenium has been shown to inhibit IL-1β-inducible nitric oxide synthases (iNOS) and cyclooxygenase 2 (COX2), matrix metalloproteinase 1 (MMP1) and 13 (MMP13) gene expressions in primary chondrocytes. These reductions in gene expression were associated with corresponding reductions in nitric oxide (NO) and prostaglandin E2 (PGE_2) production and total MMP activity. Selenium partially inhibited the IL-1β-induced phosphorylation of p38 MAPK that is commonly involved in IL-1β cell signaling.

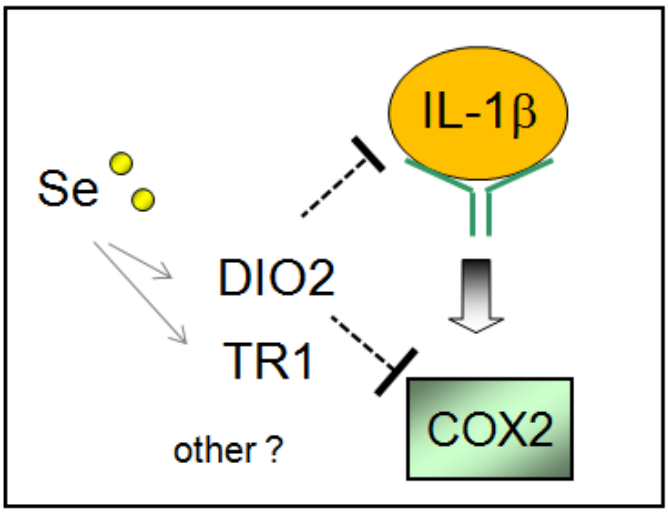


Figure 5.3 Selenoproteins modify the IL-1β-induced inflammatory response in chondrocytes. Depletion of type II iodothyronine deiodinases (DIO2) and thioredoxin reductase 1 (TR1) blocked the inhibitory effect selenium on IL-1β-induced COX2 gene expression. Our data showed that depletion of DIO2 also elevated IL-1β-induced IL-1β gene expression. DIO2 (or T3, the enzymatic product of DIO2) may negatively regulate IL-1β-induced COX2 gene expression in part through regulating IL-1β gene expression itself.

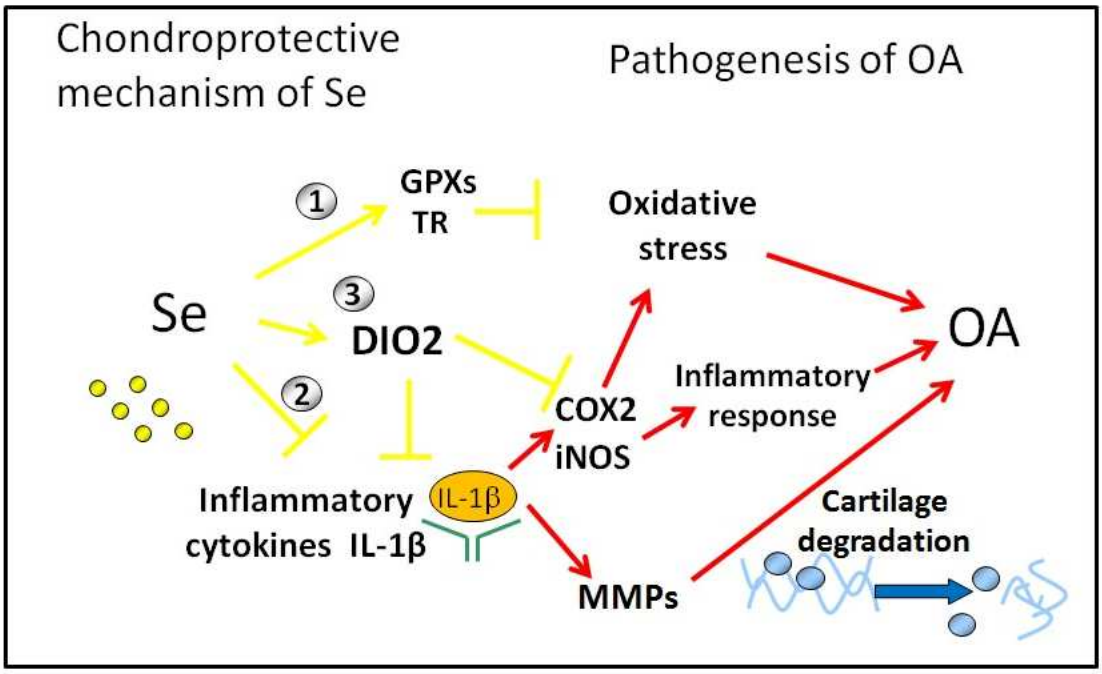


Figure 5.4 Summary of chondroprotective mechanisms of selenium. 1) Selenium induces GPX and TR activities to protect chondrocyte against oxidative stress. 2) Selenium neutralizes the inflammatory responses induced by cytokines. 3) Selenium exerts its protective effect through selenoproteins such as TR1 and DIO2 in modifying the response of chondrocytes to inflammatory stimuli.

5.3 Future Directions

Although our data suggest that DIO2 modifies the inflammatory response stimulated by IL-1β, the molecular mechanism is still unclear. It will be of interest to elucidate the cellular signaling pathways involved in the DIO2-mediated effect. In particular, thyroid hormone has been shown to positively regulate Liver X Receptor (LXR) alpha expression (Hashimoto, Matsumoto et al. 2007) and activation of LXR-decreased basal and IL-1β-induced PGE_2 production in human cartilage explants in another study (Li, Rivera-Bermudez et al. 2010). It will be interesting to test whether DIO2 or thyroid hormone regulates LXR expression and hence modify IL-1β-induced COX2 gene expression in chondrocytes.

Local availability of thyroid hormone, maintained by DIO2, is critical for growth plate formation during skeletal development and deregulation of local thyroid hormone availability has been suggested to predispose individuals to OA (Meulenbelt, Min et al. 2008; Meulenbelt, Bos et al. 2010). However, the role of DIO2 in the progression of OA, especially in the late stages of the disease, is still unclear. We demonstrated that depletion of DIO2 increased the inflammatory response of IL-1β in chondrocytes. This suggests a protective role of DIO2 based on the catabolic role of IL-1β in pathogenesis of OA. It will be informative to evaluate the impact of depleting DIO2 in articular cartilage in OA progression in an adult animal model of OA.

Our data demonstrated an anti-inflammatory effect of selenium on chondrocytes in an explants culture system. It will be informative to test if selenium treatment has a similar effect in neutralizing the catabolic and inflammatory effect of IL-1β in isolated cartilage explants or chondrocytes in alginate beads, in which response of chondrocyte is similar to that in the cartilage.

Given the fact that too little or too much selenium can be toxic, the form and the dose of selenium we defined in our *vitro* chondrocyte system can serve as a reference for preclinical animal studies. It will be interesting to determine if a selenium deficient diet would increase the severity of OA and whether a super-physiological but non toxic diet can slow or improve the manifestations of OA in an animal model such as Dunkin-Hartley guinea pig which develops OA in a pattern similar to human. We could further examine the gene expression of key selenoproteins such as GPX1 and DIO2 in the cartilage of animals under different selenium diets and determine the effect of selenium supplementation on selenoprotein expression levels and whether this provides a protective effect against OA progression. In addition, we could test whether intra-articular injection of selenium alone or combined with other therapeutics into the affected joints of an animal model, with OA induced either chemically or surgically, could relieve the symptoms based on the potential anti-inflammatory effect of selenium on pro-inflammatory cytokines such as IL-1β. Finally, antioxidative selenoproteins such

as GPX1 and TR1 as well as thyroid producing selenoproteins like DIO2 could be targets for gene therapy in the OA affected joints.

In this study, we identified DIO2 as one of the mediators of the anti-inflammatory effect of selenium in chondrocytes. Our findings corroborate a genetic study that showed a common coding variant of DIO2 was associated with increased risk for developing symptomatic OA (Meulenbelt, Min et al. 2008). It would be valuable to examine the possible association between the identified genetic variant of the DIO2 gene and levels of pro-inflammatory cytokines such as IL-1β, and biomarkers related to joint inflammation such as cartilage oligomeric matrix protein (COMP) and high-sensitivity C-reactive protein (hs-CRP) (Vilim, Vytasek et al. 2001; Sturmer, Brenner et al. 2004).

Our data suggested DIO2 may modify chondrocytes' response to IL-1β or other inflammatory responses. It would be interesting to establish the relationship between the endogenous levels of DIO2 in OA cartilage or chondrocytes and the corresponding response to IL-1β *in vitro*. Furthermore, it would be of interest to determine if the reported DIO2 polymorphism modifies the response of chondrocytes to IL-1β by examining the response of chondrocytes or synovial cells carrying specific DIO2 variants to IL-1β. The resulting data could be beneficial to predict the response of patients to anti-IL-1β treatment such as interleukin 1 receptor antagonist (IL-1RA) based on the information of DIO2 polymorphism.

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Publications
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Conference Abstract

1. **Cheng, A. W**., Stefano J. E., Li, D., Kyzaike, J., Boudanova, K., Qiu, H., Hou, L., O'Callaghan, M., Kraus, V.B., Mathews. G.L. Characterization of Sustained Release Native and Modified Human sFlt01 formulation for intraarticular delivery to treat OA pain. Osteoarthritis and Cartilage 2010: 18: S240-S241
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