EFFECTS OF LUTEIN AND β-CRYPTOXANTHIN ON MMP-13, PGE$_2$, AND

CYTOKINE PRODUCTION IN HUMAN CHONDROSARCOMA CELLS INDUCED WITH

INTERLEUKIN-1β

By

MICHAEL M. DI FILIPPO

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To the Faculty of Washington State University:

The members of the committee appointed to examine the thesis of MICHAEL M. DI FILIPPO find it satisfactory and recommend that it be accepted.

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Boon P. Chew, Ph.D., Chair

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Phil Mixter, Ph.D.

_____

Joe Powers, Ph.D.

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Abstract

By: Michael M. Di Filippo, M.S.
Washington State University
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Chair: Boon P. Chew

Osteoarthritis (OA) and rheumatoid arthritis (RA) are characterized by the activation of matrix-degrading proteinases and production of inflammatory cytokines and mediators by chondrocytes in articular cartilage. Several studies have shown that the carotenoids lutein (Lu) and β-cryptoxanthin (βCr) may be useful in downregulating factors involved in the progression of OA and RA Therefore, the possible protective effects of Lu and βCr against arthritis were studied using SW-1353 cells induced by interleukin-1β (IL-1β). Cells were cultured for 24 hr with 0, 0.01, 0.1 and 1.0 μmol/L of Lu (Experiment 1) or βCr (Experiment 2) and stressed by IL-1β for 24 hr. Conditioned medium was analyzed for proteinases, inflammatory cytokines and mediators, and nuclear extract for NFκB. The addition of βCr had no effect on matrix-metalloproteinase-13 (MMP-13) production; however, 1.0 μmol/L Lu decreased matrix MMP-13 ($P < 0.02$) and PGE$_2$ ($P < 0.04$) production. Similarly the addition of 0.1 and 0.01 μmol/L βCr also decreased ($P < 0.01$) PGE$_2$ production. At various concentrations (0.01, 0.1, and 1.0 μmol/L), βCr decreased the pro-inflammatory cytokines IL-1α ($P < 0.05$), IL-2 ($P < 0.04$), and IFN-γ. In contrast, Lu increased the IL-1α, IL-2 ($P < 0.01$), and interferon (IFN)-γ. Lu increased the anti-inflammatory cytokines IL-4 ($P < 0.02$) and IL-10, while βCr decreased IL-4 and IL-10 ($P < 0.02$).

NFκB p50 was decreased by Lu at all concentrations tested including a 39% reduction in cultures containing 0.1 μmol/L. The βCr also decreased concentrations of nuclear p50 by 38% in cells preincubated with 1.0 μmol/L. In conclusion, Lu and βCr protected against degenerative factors upregulated by IL-1β-stimulation, likely by scavenging reactive oxygen species required for NFκB activation. Chondrocytes cultured in the presence of Lu downregulated factors involved in cartilage destruction (MMP-13 and PGE$_2$) and upregulated factors (IL-4) involved in tissue-protection, while addition of βCr resulted in strong downregulation of factors associated with pro-inflammatory response (IL-1α, IL-2, and IFN-γ) and cartilage destruction (PGE$_2$).

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LITERATURE REVIEW

1. Arthritis: Introduction

1.1. Prevalence. According to the Center for Disease Control (CDC), there are approximately 100 different diseases and conditions that arthritis encompasses. Osteoarthritis (OA) is the most prevalent of these diseases and has been conservatively estimated to have affected almost 27 million US adults in 2005; this is an increase from 21 million in 1990 (Lawrence et al. 2008). Other common arthritic diseases and conditions include fibromyalgia, gout, and rheumatoid arthritis (RA). In 2005, RA affected an estimated 1.3 million adults aged 18 and older (Helmick et al. 2008). When all arthritic diseases and conditions are taken into consideration, it is estimated there are 46 million self-reported or doctor-diagnosed US adults affected annually (Goldring 2006, Messer et al. 2003). Of this population, almost 19 million people have limitations in activity, and 36 million partake in outpatient visits (Helmick et al. 2008). Children are also susceptible to arthritis, and it has been recently estimated that 1 in 250 children have some form of arthritis (Arthritis Foundation. 1999).

1.2. Incidence. In OA, joint degeneration commonly occurs in weight bearing joints such as the knee and hip. However, it often manifests in the hands and feet. Overall, lesions in OA are usually non-symmetrical and affect only one side of the body. Of the 27 million people affected with OA, 13.9% are adults 25 years and older and 33.6% of those are over 65 (Lawrence et al. 2008). RA usually affects multiple joints in a symmetrical pattern, in which both sides of the body are affected, often including the small joints of the hands, feet, and cervical spine. Large joints such as the shoulder and knee can also become involved (Harris 2005). Data collected from the National Health and Nutritional Examination Survey (NHANES) has shown that RA is primarily present in both men and women between 35-65 years old, with 30% being over 65 (CDC 1995). Sex appears to play a key role in both OA and RA. OA manifests twice as

frequently in the hands and knees of women compared to men (CDC 1995), and women are 2.5 times more likely to have RA (Arthritis Foundation 1999)

1.3. Hospitalization. In 2004, there were 20,000 RA-related hospitalizations and 80% of those were among people over 45 years of age. In 1997, approximately 4 million ambulatory care visits took place in the US among people with RA. This constituted 10.9% of all arthritis and rheumatic diseases, with OA accounting for 55% of all arthritis-related hospitalizations (Hootman et al. 2002, Lethbridge-Cejuku et al. 2003). It was also estimated in 1997 that OA accounted for nearly 20% (7.1 million) of all arthritis-related ambulatory medical care visits (Hootman et al. 2002). Furthermore, arthritis or rheumatism, according to data from 1991-1992, is the leading cause of disability in the US among persons over 15 yr of age (CDC 2005).

1.4. Cost. The total cost attributed to arthritis and other rheumatic conditions in the US increased from $86 billion in 1997 to $128 billion in 2003. In a three-year study of 7,527 patients, the mean direct medical care cost of an RA patient was more than $9,519 per year, with drugs and hospital costs attributing 66% and 17% respectively, to the total cost (Koo et al. 2001). Increasing numbers of joint replacement procedures were performed; knee and hip joint replacements accounted for 35% of these arthritic-related procedures (Gabriel et al. 1997, Mehrotra et al. 2005). OA is the most common cause for total hip and knee replacement (Jüni P 2006). Although a single joint can be involved, it is more common for several joints to be affected (Goldring et al. 1994). OA may present a strain on the economy by accounting for $3.4 to $13.2 billion per year in job-related expenses (Buckwalter et al. 2004). The cost of these procedures was estimated at $7.9 billion in 1997 (Helmick et al. 2008), and it is estimated that patients pay an average of $2,600 for treating OA each year (Michaud et al. 2003).

OA and RA are not only the most common of arthritic diseases but also the most similar in that they both involve progressive degeneration and loss of articular cartilage leading to debilitating impairment of joint motion and function (Mazzetti et al. 2001).

2. Rheumatoid Arthritis

Rheumatoid arthritis is a chronic autoimmune disease characterized by the localization of inflammatory cells in the synovial lining, leading to joint erosion and progressive bone and articular cartilage destruction (Kim et al. 1998).

2.1. Etiology. The etiology of RA is unknown (Sango 2000); however, several risk factors are associated with the disease. These include sex hormones, menstrual and reproductive factors, genetics, age, socio-economic status, education, stress, smoking, environment, and infectious agents (Lethbridge-Cejuku et al. 2002, Arnett et al 1988). Unlike OA, RA is considered a multifactorial disease resulting from environmental and genetic factors (Goldring et al. 1986). Studies of monozygotic twins and dizygotic twins have suggested the influence of genetic factors (Aho et al. 1986, Silman et al. 1993). Genetic markers have identified a relationship between RA and a shared epitope on small regions of the DRB1*0401 and *0404 alleles (Wordsworth et al. 1989). Further analysis has suggested an association between HLA alleles and rheumatoid factor, nodules, and erosion (Seldin et al. 1993).

2.2. Diagnosis. The current criteria for diagnosing RA are based on the 1987 American Rheumatology Association (ARA) classification. Diagnosis is based on clinical, laboratory and radiological abnormalities. The criteria include morning stiffness, arthritis of at least 3 affected areas, arthritis of hand joints, symmetrical arthritis, rheumatoid nodules, serum rheumatoid factors, and radiographic changes (Arnett et al. 1988). Morning stiffness is classified as stiffness

lasting longer than 1 hr and of at least 6 wks duration. Arthritis of at least 3 affected areas refers to soft tissue swelling or exudation lasting more than 6 wks. Arthritis of the hand joints includes the wrist, metacarpophalangeal (MCP), and proximal interphalangeal (PIP) joints in which arthritis lasts beyond 6 wks, and the criteria for symmetrical arthritis is occurrence in at least one area for more than 6 wks. Rheumatoid nodules are determined by a physician and serum rheumatoid factors are assessed clinically. Finally, radiographic changes are seen on anteroposterior films of wrists and hands. The accuracy of these criteria in diagnosing RA and distinguishing it from other rheumatic conditions shows a specificity of 89% and sensitivity between 91% and 94% (Arnett et al. 1988).

2.3. Pathology. The process of joint degradation in RA begins with inflammation of the synovial tissue (Fassbender 1994). Normally, the synovium functions to line the joint cavity, provide nutritional support to articular cartilage, and lubricate cartilage surfaces (Otero and Goldring 2007). The initial inflammation in RA stems from the synovial lining of two opposing bone surfaces covered by cartilage (Mehrotra et al. 2005). The inflammation in these joints is characterized by proliferation of cells lining the synovium, and increased vascularization. The amplified presence of blood vessels in this area is accompanied by the migration of multiple cells, including B-cells, T-cells (CD4+), phagocytes, type A (macrophage-like) cells, type B (fibroblast-like) cells, and neutrophils (Mehrotra et al. 2005). This is clinically evident by joint swelling and pain (Buckwalter et al. 2004). In the more chronic phase of RA, a lesion proceeds to develop as an overgrowth of tissue mass called the pannus. The distal end of the proliferating pannus structure invades the extracellular matrix of cartilage (Takasugi and Inoue 1988), and degradation of extracellular matrix is mainly due to cells of the pannus expressing massive amounts of messenger RNA (mRNA) encoding for matrix metalloproteinases (MMPs)

(Buckwalter et al. 2004). A similar destructive process occurs at the interface between the synovium and the bone, and as a result the invading pannus penetrates the joint space, which initiates local activation of bone resorption and destruction of the mineralized bone matrix (Mehrotra et al. 2000). It is evident that inflammation plays a key role in the pathogenesis of RA. However, when pain and inflammation are closely controlled with anti-inflammatory agents, the process of joint destruction continues, including both cartilage degradation and bone erosion. In this absence of inflammation, cytokines and mediators appear to be the driving force as shown in research utilizing radiographs of persons with RA (Goldring et al. 1994). RA affects not only the joint, but also frequently involves extra-articular organs as shown in **Table 1** (Lee and Weinblatt, 2001).

3. Osteoarthritis

OA is a musculoskeletal disorder that involves the degeneration of articular cartilage, intra-articular inflammation with synovitis, and alterations in peri-articular and subchondral bone (Dieppe and Lohmander 2005).

3.1. Etiology. Although the etiology of OA is unknown, many factors have been associated with the progression of the disease (Marks and Allegrante 2007). These factors can be grouped into three categories; mechanical injury, hereditary factors, and aging (Harris 2005, Marks and Allegrante 2007), and may include muscle weakness, joint instability and/or malalignment, obesity, associated intra-articular crystal deposition, peripheral neuropathy, aging, oxidative stress, bone abnormalities, fractures and diseases, decreased birth weight, fall injuries, gender, joint deformity, leg length discrepancy, joint laxity, joint surgery, occupational stresses,

sports, and motor vehicle injuries. Also included are systematic factors such as bone density, and trauma.

Table 1. Extra-articular involvement of rheumatoid arthritis (Dieppe and Lohmander 2005)

Organ system	Involvement
Skin	Rheumatoid nodules, vasculitis
Ocular	Keratoconjuctivitis sicca, iritis, episcleritis
Oral	Salivary inflammation (sicca symptoms)
Respiratory	Pulmonary fibrosis, pleural effusion. Cricoarytenoid inflammation
Cardiac	Pericardial inflammation, valvular nodule formation, myocarditis
Neurological	Mononeuritis, nerve entrapment, cervical instability
Hepatic	Increased aminotransferase concentrations
Haematological	Anemia, thrombocytosis, leucocytosis, lymphadenopathy, Felty's syndrome: splenomegaly, thrombocytopenia
Vascular	Vasculitis

3.2. Diagnosis. Diagnosis of OA is relatively easy, and is identified by several common clinical features, including increased age, pain, stiffness, reduced movement, swelling, and crepitus as outlines by the ARA (Lawrence et al. 2008). The usual onset of OA occurs primarily

in people over 40 years. Most people with OA develop short-duration stiffness during inactive periods, usually lasting only minutes. Joint movement is often restricted due to general pain with movement. Osteophyte formation leads to the development of palpable, firm swelling at the joint margin. Secondary synovitis accounts for minor soft tissue swelling. Crepitus is common in OA joints which crack or creak during movement (Lawrence 2008). The most challenging aspect of clinical diagnosis of OA is differentiating it from other common regional joint pain, including referred pain, periarticular (soft-tissue) conditions, and somatisation (regional pain in the absence of any defined pathological cause). Diagnosing the severity of OA involves an assessment of both the joint and patient. Joint examination is used to examine damage (deformity and instability) and radiographic examination is often used to support the diagnosis of OA (Lawrence 2008).

3.3. Pathology. The pathology of OA can be classified as a whole joint disease involving the loss of focal and progressive hyaline articular cartilage. Affiliated alterations in bone underlying the cartilage include marginal outgrowths, osteophytes, and increased thickness of subchondral bone. Structures around the joint that are affected in OA include the synovium, ligaments, and bridging muscles (NAAP 1999). The synovium may directly participate in joint degeneration while ligaments and bridging muscle are likely victims of joint instability and degeneration.

Early stages of OA are characterized by inflammation, repetitive injury, and subchondral bone changes. Initiation of these changes is believed to be caused by imbalances in expression, activity and signaling of cytokines and growth factors (Lethbridge-Cejuku et al. 2003). Hallmarks of early OA are Golgi cluster formation, increased MMP production, a disintegrin and metalloproteinase with a thrombospondin type 1 motif (ADAMTS) production, cytokine

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production, proteoglycan synthesis/degradation, tidemark disruption/angiogenesis, collagen degradation, and release of matrix fragments. Hallmarks of progressive and later stages of OA include cell senescence, cell apoptosis, collagen gene activation, altered collagen types, hypertrophy, osteophyte formation, and tidemark duplication (Lethbridge-Cejuku et al. 2003). The pathogenesis of joint damage in OA can generally be divided into three regions: the synovium, bone, and cartilage.

OA is technically classified as a non-inflammatory disease as defined by low synovial fluid leukocyte counts of less than 2000 cells/mm (Goldring 2006, Kim et al. 1998). Clinically, the joint of OA patients contraindicates this phenomenon, evidenced by swelling, effusions, and stiffness. These signs and symptoms clearly reflect synovial inflammation as a low-grade contribution to disease pathogenesis (Koo 2001). In OA, as opposed to RA, synovial inflammation is localized in areas adjacent to pathologically damaged cartilage and bone. This proximity allows the synovium to release proteinases and pro-inflammatory cytokines and mediators that accelerate the degradation of nearby cartilage (Koo 2001). In a recent study, synovial tissue from 10 early OA patient arthroscopic specimens showed increased levels of interleukin-1-β (IL-1β) and tumor necrosis factor-α (TNF-α) when compared to 15 patients with late OA undergoing total knee arthroplasty (Benito et al. 2005). Another study reported elevated levels of proteinases in patients with destructive hip OA, evident by increased MMP-3, and MMP-9 concentrations in the synovial cells, synovial fluid, plasma, and sera (Masuhara et al. 2002, Tchetverikov et al. 2005). This increase in degradative proteinases is a likely response to increased inflammatory cytokines and mediators.

The role of bone in OA is exemplified by the presence of osteophytes and thickening of subchondral bone, best observed with MRI. The role of pro-inflammatory synovial cytokines and

mediators in the pathogenesis of bone is not well understood (Goldring et al. 1994). Nitric oxide (NO) may play a role in subchondral bone changes in OA. Endothelial cell nitric oxide synthase (ecNOS) and inducible nitric synthase (iNOS) are both expressed in bone cells. It is likely that ecNOS regulates osteoblast activity and bone formation while mediating the effects of mechanical loading on the skeleton. In addition, iNOS, which is likely induced by IL-1β and TNF-α in bone cells, leads to increased NO concentrations which appeared to facilitate bone loss (Goldring et al. 1994, Pelletier et al. 2001). Osteophyte formation and subchondral bone remodeling are the result of local production of anabolic growth factors such as insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β) (Bettica et al. 2002).

The pathogenesis of OA is different from RA. OA originates in the cartilage and may spread, in advanced cases, to synovial tissue encompassing the joint, while RA begins in the synovial tissue adjacent to the joint before spreading to cartilage (Pashkow et al. 2008). However, the activation of matrix-degrading proteinases and the production of inflammatory cytokines and mediators, specifically those produced by chondrocytes in articular cartilage, are very similar.

4. Cartilage destruction

    4.1. Chondrocytes. Chondrocytes exclusively reside in cartilage, and in the joint are non-uniform, and vary in size, shape, and concentration depending on the different zones of the cartilage. They are found within an extracellular matrix consisting of types II, IX, and XI collagen and providing tensile strength and preservation of proteoglycans (Buckwalter et al. 2004, Horton et al. 1998). The formation, maintenance and turnover of various extracellular matrix proteins are an exclusive responsibility of chondrocytes (Arnett et al. 1988). In OA,

chondrocytes react to adverse environmental stimuli by promoting cartilage degradation (primarily type II collagen) and downregulation of processes critical for cartilage repair (Koo et al. 2001). In RA, chondrocytes participate in cartilage degradation (primarily type II collagen) in transitional and deep zones by degrading their own matrix through autocrine and paracrine mechanisms. They are also involved in the loss of proteoglycans in the transitional and deep zones of cartilage (Buckwalter et al. 2004). Activation of degrading proteinases is mainly regulated by inflammatory cytokines such as IL-1β.

    4.2. Inflammatory cytokines. IL-1β is a potent pro-inflammatory cytokine that affects many cell types throughout the body. Research has shown that IL-1β is the key force in promoting an imbalance between cartilage degradation and repair. In chondrocytes, IL-1β promotes the synthesis and release of MMPs, pro-inflammatory cytokines and mediators which contribute to the pathogenesis of OA and RA (Badger et. al, 1999, Shikhman et al 2001).

    Human chondrocytes stimulated with IL-1β *in vitro* have been shown to increase the production of MMPs and several inflammatory cytokines and mediators (Arnett et al. 1988, Guillén et al. 2008, Panico et al. 2006) such as MMP-1 and MMP-13, TNF–α, NO and prostaglandin E_2 (PGE_2). The NO and PGE_2 are synthesized by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively (Schuerwegh et al. 2003, Schulze-Koops and Kalden 2001). Other important pro-inflammatory cytokines, IL-1α, IL-2, IL-6, IL-8, interferon-γ (IFN-γ) may also be elevated. In addition, the anti-inflammatory cytokines IL-4 and IL-10 may be involved in protective mechanisms. Collectively, pro-inflammatory and anti-inflammatory cytokines are characterized using CD4+ Thelper (Th) cell differentiation. CD4 Th can differentiate into two different subsets, Th1 and Th2, based on cytokine stimulation. Th1 cells are mainly activated by inflammatory cytokines. They promote cellular immunity and are

involved in autoimmune diseases by secreting inflammatory cytokines that promote the proliferation of macrophages, NK cells, B cells, and T cells. The Th2 cells are mainly activated by anti-inflammatory cytokines. They promote humoral immunity and mediate allergic immune responses by secreting inflammatory cytokines that promote the proliferation of B cells, mast cells, and eosinophils (Schulze-Koops et al. 2001). Chondrocytes can alter the Th1/Th2 balance, depending on the type and amount of cytokines secreted. Therefore, chondrocytes stimulated with IL-1β are expected to produce pro-inflammatory cytokines that may lead to Th1 cell response. Furthermore, these Th1 cells can inhibit the expansion of Th2 cells, and likewise Th1 inhibition by Th2 cells. Although the relationship between Th1 and Th2 cell activation has mainly been studied with RA, the type and amount of Th1/Th2 cytokines present in OA are very similar as well.

The pro-inflammatory cytokine TNF-α has been found in high concentrations in OA and RA tissues and is also believed to play a key role in the progression of these conditions. TNF-α functions to stimulate the production of pro-inflammatory mediators and catabolic proteinases in cartilage (Fernandes et al. 2005). Inhibition of TNF-α has been shown to inhibit cartilage degradation in OA cartilage. PGE_2 may also play a role in the progression of cartilage matrix degradation by promoting the production of MMPs (Hardy et al. 2002). It has also been reported that PGE_2 enhances the vulnerability of human chondrocytes to NO-induced apoptosis and induces inflammation by increasing blood flow and enhancing the effects of IL-1β on vasopermeability (Amin et al. 1998, Smith et al. 1998).

4.3. Matrix metalloproteinases. Matrix metalloproteinases are a family of enzymes capable of degrading proteins of the extracellular matrix (Belotti 1999). They are involved in tissue remodeling in both physiological and pathological processes. The MMP family of

proteinases includes four interstitial members: collagenase-1 (MMP-1), collagenase-2 (MMP-8), collagenase-3 (MMP-13) and membrane-type-1 MMP (MMP-14) (Raymond et al. 2007). The up-regulation of MMPs has been strongly implicated in both OA and RA.

Synovial cells and chondrocytes produce several MMPs which have the ability to degrade all components of the extracellular matrix including collagens and proteoglycans (Buckwalter et al. 2004, Julovi et al. 2004). The upregulation of MMP-3 results in proteoglycan loss, thereby reducing cartilage stiffness (Kempson 1979). In OA and RA, MMPs mainly function to degrade the collagen present in cartilage (Julovi et al. 1997), and the presence of specific MMPs is determined by the location, cell type and pathogenic process of the condition. MMP-14 is only expressed in RA synovium while MMP-1, MMP-8 and MMP-13 are expressed in both synovium and cartilage of OA and RA patients. MMPs expressed by chondrocytes during inflammation include MMP-1 and -13. MMP-13 is most active against type II collagen in cartilage (Knäuper et al. 1997). It has been shown that MMP-1 and MMP-13 are key enzymes in the initiation of OA as well as RA, as both enzymes are expressed in cartilage lesions before the onset of clinical disease (Tchetina et al. 2005). Furthermore, MMP-1 and-13 are rate-limiting in the process of collagen degradation (Burrage et al. 2006). Down-regulating MMP production may halt the progression of joint degeneration common in OA and RA. The secretion of MMPs by both adjacent synovial tissue and chondrocytes is mainly mediated by IL-1β in an autocrine or paracrine fashion (Mankin and Brandt 1997, Marks and Allegrante 2007). Induction of chondrocytes by IL-1β results in increased cellular concentrations of reactive oxygen species (ROS), leading to the upregulation of transcriptional factors necessary for adequate generation of MMPs.

4.4. Reactive oxygen species.

ROS are highly reactive ions or small molecules that have unpaired valence shell electrons present. The ROS family includes oxygen ions, free radicals, and peroxides. ROS present in chondrocytes include hydrogen peroxide (H_2O_2), hypochlorite ion (OCl^-), hydroxyl radical ($\cdot OH$), superoxide anion (O_2^-), and NO (Henrotin and Kurz 2007). The main ROS produced by chondrocytes are O_2^- and NO, along with their derivative radicals such as peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2) (McChartney-Francis et al. 1993). ROS are unavoidable byproducts of cellular respiration and are essential for cell signaling. Neutrophils and macrophages also produce ROS for the phagocytic killing of some types of bacteria. Unfavorable consequences resulting from the presence of ROS involves the ability of ROS to interact with other molecules to gain stable electron conformation . These highly reactive molecules can destroy cellular membranes, proteins, and nucleic acids (Chew and Park. 2004).

ROS, particularly NO, have been found in high concentrations in synovial fluid of patients with OA and RA (Farrell et al. 1992). Early studies have shown a reduction of symptoms and protection against joint injury through inhibition of iNOS expression (Ianenti et al. 1993, McCartney-Francis et al. 1993). Excessive amounts of NO may induce MMP synthesis (Murrell et al. 1995). Research by Pelleteir et al. (1996) has shown the ability of NO to reduce IL-1Ra production, leading to an increased progression of cartilage matrix degradation by IL-1. The ability of IL-1-β to induce the ROS production has been well-documented in the pathogenesis of OA and RA (Lo et al. 1996). In response to stimuli such as IL-1β, ROS production greatly increases leading to oxidative stress. This overwhelming ROS attack can consequently activate cell signaling pathways involved in inflammation and joint remodeling

(Pashkow et al. 2008). Recent studies have emerged spotlighting the role of ROS in tissue damage (Arnheim and Cortopassi 1992, Dumont et al. 2000, Finkel and Holbrook 2000, Toussanit et al, 2000).

High concentrations of ROS, and therefore oxidative stress, can lead to premature senescence and apoptosis of chondrocytes (Blanco et al. 1998). Senescence and apoptosis characteristically occur in later stages of OA and RA. Cell senescence is likely a result of shortened telomeres, along with decreased mitochondrial ATP production and increased concentrations of β-galactosidase (Martin et al. 2004). These events have been validated histologically in chondrocytes removed from OA patients (Kuhn et al. 2004). Senescence may specifically be caused by oxygen free radicals which directly damage guanine repeats on the telomere DNA, leading to telomere erosion (Yermilov et al. 1995). Oxidative stress not only effects chondrocyte telomere length but also cellular replication lifespan, function, cartilage matrix proteoglycan structure, and composition *in vitro* and *in vivo* (Yudoh et al. 2005). Chondrocyte apoptosis may be a direct result of high NO concentrations, commonly seen in both OA and RA (Lethbridge-Ceijku et al. 2003). This correlates with increased apoptosis and iNOS protein in articular cartilage cells (Kim et al. 1998). Canine and murine models have also been used to examine the effect of reduced progression of cartilage lesions and NO inhibition (Pelletier et al. 1998). Several other studies have similarly demonstrated that NO, ROS, are involved in the pathogenesis of OA and RA (Del Carlo and Loeser 2002, Pelletier et al. 2000, Sadler et al. 1991, Studer et al. 1999).

ROS can also cause cellular damage to chondrocytes leading to the release of cellular contents into the extracellular surrounding. Intracellular contents are mainly composed of oxidized molecules that can trigger neighboring chondrocyte and synovial cells to release

inflammatory cytokines and mediators, MMPs, and additional ROS (Blanco et al. 1995, Del

Carlo and Loeser 2002, Henrotin et al. 2003). ROS damage to cartilage has been demonstrated

by measuring the presence of lipid peroxidation products (lipid hydroperoxides and aldehydes)

and nitrotyrosine *in situ* (Loeser et al. 2002). Research has also shown the presence of elevated

nitrated type II collagen peptides in sera of patients with OA, which is indicative of $ONOO^-$

formation in OA cartilage (Deberg et al. 2005). Several studies have implicated ROS in IL-1-

mediated cellular responses (Eberhardt et al. 2002, Kuo et al. 1997). Inhibition of ROS may

decrease production of MMP and pro-inflammatory cytokines and mediators by IL-1β-induced

chondrocytes. This could lead to decreased production of MMP-13, pro-inflammatory cytokines,

PGE_2, and NO, and an increase in anti-inflammatory cytokines.

`   The upregulation of transcriptional factors involved in collagen degradation and

inflammation has been well studied. In OA and RA, IL-1β greatly contributes to elevated

expression of MMPs and pro-inflammatory cytokines and mediators. IL-1β stimulation of

chondrocytes both *in vitro* and *in vivo* activates transcriptional pathways, activator protein 1 (AP-

1) and nuclear factor κ B (NF-κB) **(Figure 1)** (Aho et al. 1986, Burrage et al. 2006, Martel-

Pelleteir 2001, Mengshol et al. 2000 Tetlow et al. 2001).

IL-1β △

IL-RI, IL-1RAcp

IRAKS, MyD88

?

TRAF-6

P NIK ← P TAK 1

MEK K 1,3

MEK 1,2

P IKK

P MKK 3,6

P MKK 4

P ERK 1,2

P IkB

p50 p65

P p38

P JNK

P ELK-1

P ELK-1 → c-fos

P ATF-2

P c-jun

c-jun

Cytoplasm

Nucleus

p50 p65

NFκB

P □ ○ P AP-1

Pro-inflammatory cytokines, iNOS, PGE$_2$, COX-2, MMP-13
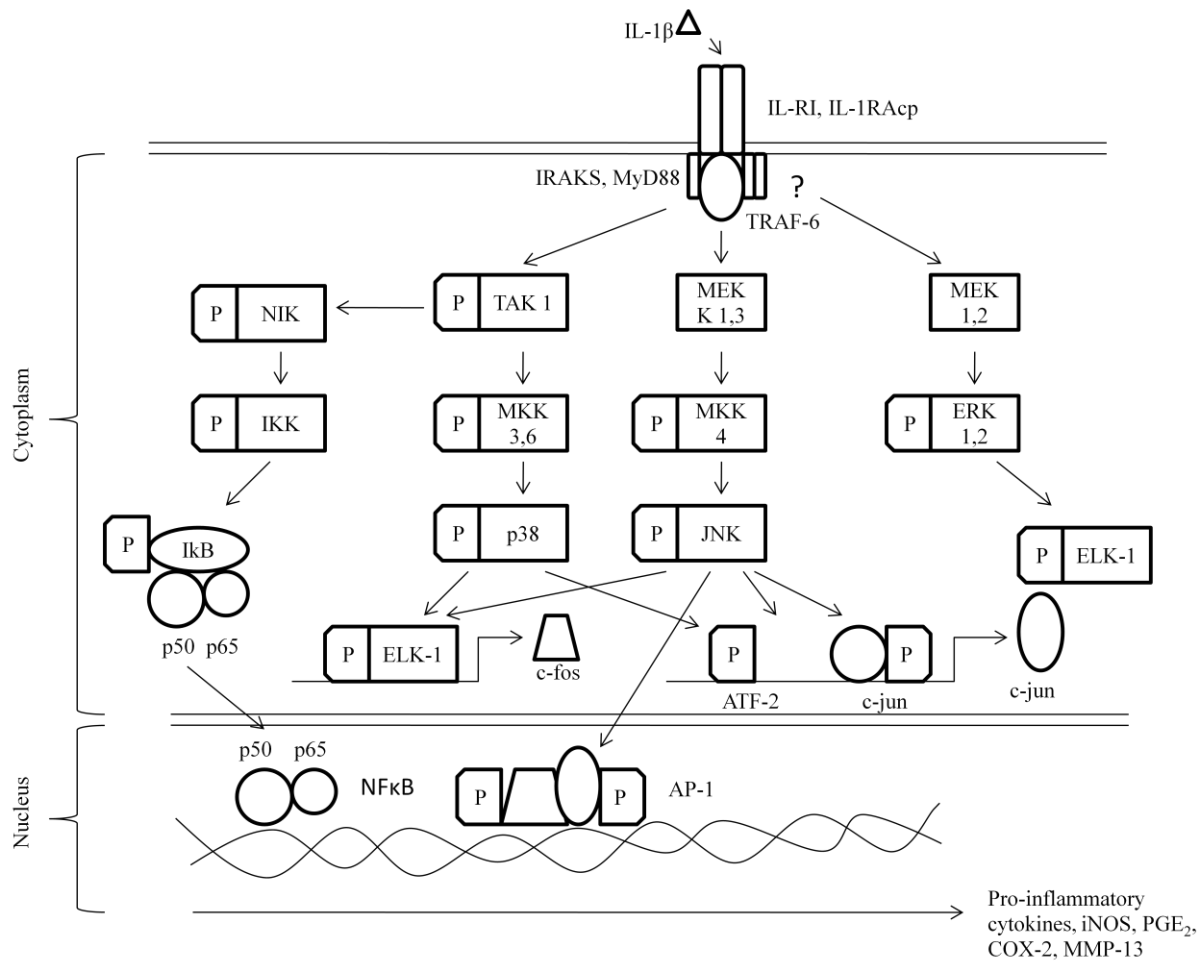
Figure 1. Interleukin 1β induced transcriptional pathway (Badger et al. 1999)

5. Cellular models for studying arthritis

There are several models commonly used for studying chondrocyte involvement in OA and RA pathogenesis, as well as IL-1β-induced MMP for the production of cytokine, and mediator. These models include primary cultures of chondrocytes obtained from young animals, primary human chondrocytes (PHC) obtained from healthy and diseased individuals, immortalized cell lines from these primary cultures, and chondrosarcoma cell lines..

5.1. Primary cells. In a study using IL-1β-induced human OA chondrocytes obtained from the Cooperative Human Tissue Network, significant increases in NO and PGE_2 were shown to be caused by upregulation of iNOS and COX-2, respectively (Ahmed et al. 2002). Green tea polyphenol epigallocatechin-3-gallate (EGCG) significantly inhibited IL-1β-induced COX-2 and iNOS expression in OA chondrocytes, likely through its antioxidant activity (Burrage et al. 2006). Human arthritic chondrocytes obtained from femoral head cartilage and normal human chondrocytes have been shown to significantly increase MMP-1, MMP-3 and MMP-13 production when treated with 2 ng/ml of IL-1β (Kempson 1979). Experiments using normal articular chondrocytes have shown similar results. In normal chondrocytes, 10 ng/ml of IL-1β significantly induced the production of NO and PGE_2.The production of inflammatory mediators was significantly suppressed with the addition of methanolic extracts from the flowering buds of *Capparis spinosa* L (LECS), thereby counteracting the harmful effects of   IL-1β stimulation (Panico et al. 2005). It was postulated that the inhibitory effect of LECS may be due in part to the flavonoids present. These compounds act as antioxidants and may be involved in ROS scavenging, leading to the downregulation of NO and PGE_2. These studies highlight the effects of both IL-1β and antioxidants in joint degradation in OA and RA. However, the study failed to determine the direct mechanism by which antioxidants such as EGCG and LECS inhibit IL-1β-induced production of MMPs, cytokine, and mediators. Furthermore, the use of PHC has been problematic due to low availability of cartilage, low yield of chondrocytes from surgical procedures, differentiating phenotype when expanded in monolayer culture, and variability between donors and preparations (Goldring et al. 1994, Finger et al. 2003). Also, proliferation of PHC *in vitro* is associated with loss of common morphology and gene expression. These

difficulties have led to an interest in using immortalized chondrocyte cell lines for studying joint degradation in OA and RA.

5.2. Immortalized cell lines. The use of immortalized human chondrocytes has been met with mixed results. Treatment of human chondrocytes with the immortalizing antigen, SV40 large T, resulted in an increase in genes involved in cellular proliferation at the expense of those which regulate matrix turnover and formation (Finger et al. 2004), resulting in loss of phenotype. This is evident with the immortalized chondrocyte cell line, C-20/A4, that shows little response to IL-1β with regards to MMP production seen with PHC. This cell line shows an inability to express extracellular matrix genes, which is consistent with the lack of MMP production (Deberg et al. 2005, Loeser et al. 2002).

5.3. Chondrosarcoma cell lines. The use of the chondrosarcoma cell line, SW-1353, available from the American Type Culture Collection (ATCC) has eliminated many of the problems associated with PHC and immortalized cell lines. Extensive research has compared PHC and SW-1353 cells regarding gene expression analysis. Results show that the SW-1353 cell line is a suitable model for examining pathological factors in OA and RA. The SW-1353 cells have strong similarities to PHC with respect to catabolic effects after treatment with 1.0 ng/mL IL-1β, including activation of the transcriptional regulator NFkB and subsequent production of MMP-1, MMP-3, and MMP-13 (Gebauer et al. 2005). IL-1β-induced SW-1353 cells can also activate the three MAPK pathways p38, ERK, and JNK, leading to downstream activation of the transcriptional factor AP-1 (Tetlow et al. 2001, Eder. 1997). It has recently been shown that p38 regulates IL-1β stimulation of both MMP-1 and MMP-13 (Kempson 1979). This is consistent with previous reports of a dose-dependent decrease in the production of MMP-13 and MMP-1

when SW-1353 cells were treated with p38 kinase inhibitor (Pei et al. 2006). The SW-1353 cells certainly are a valuable cell line for investigating IL-1β-induced joint degeneration.

6. Antioxidant function

Currently, treatment of OA and RA rely on non-surgical interventions, mainly pharmacological interventions such as the use of analgesics (acetaminophen or paracetamol), non-steroidal anti-inflammatory drugs (NSAIDs), as well as intra-articular injections of steroids and hyaluronans. Unfortunately, NSAIDs, analgesics, and various intra-articular injections mainly function to relieve symptoms of the disease while failing to address the degenerative processes underlying the damage occurring between the joint space and the bones (Gaby et al. 1999, Rashad et al. 1989, Qvist et al. 2008).

Antioxidants may play a beneficial role in treating the pathogenesis and symptomatic manifestations seen in OA and RA by decreasing cartilage degradation, synovial inflammation and bone erosion. Scavenging harmful ROS such as NO has been shown to down-regulate gene activation that leads to overproduction of MMPs, inflammatory mediators and cytokines.

An antioxidant is a molecule that is capable of stopping or slowing the progression of oxidative damage inflicted on cells by ROS. Antioxidants can be grouped into two major categories: endogenous and dietary.

Chondrocytes contain endogenous enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) that function to scavenge ROS. These enzymes function to maintain optimal oxidant:antioxidant balance. When these systems are overwhelmed by ROS, this optimal balance is lost, and intracellular oxidative stress occurs resulting in cellular damage and possible apoptosis. This damage leads to the pathogenesis

prevalent in OA and RA (Ianenti et al. 1993). When the enzymatic antioxidants fail to eliminate ROS, exogenous (or dietary) antioxidants are required. Because our present research interest is on the antioxidants lutein (Lu) and β-cryptoxanthin (βCr), only these carotenoids will be discussed.

7. Lutein & β-Cryptoxanthin

7.1. In Nature

Lu and βCr are naturally occurring, food-derived compounds. Lu is a yellow to orange-red pigment commonly found in a wide variety of fruits and vegetables, including spinach, kale, lima beans, broccoli, oranges, celery and string beans. However, Lu exists mainly in dark green leafy vegetables (Mangels et al. 1993). βCr is a yellow to orange-red pigment commonly found in red bell peppers, papaya, cilantro, oranges, corn and watermelon. Animals do not synthesize Lu or βCr; therefore, they must be derived from the diet. Lu, βCr, and many other carotenoids are synthesized by plants and algae from the common precursor phytoene **(Figure 2)** (Bucsi and Poor 1998). The ability of plants, bacteria, and fungi to synthesize Lu and βCr is dependent on the presence of specific enzymes involved in photosynthesis (Hirschberg et al. 1997).

The carotenoid family encompasses more than 600 pigments. This family can be divided into two important classes: the carotenes, which are composed of carbon and hydrogen, and the xanthophylls, which are oxygenated (Higuera-Ciapara et al. 2006). Lu and βCr belong to the xanthophyll class of carotenoids. Structurally, Lu and βCr are closely related to zeaxanthin and β-carotene **(Figure 3)**. Lu and zeaxanthin are unique in that it has one polar hydroxyl group in each of its two terminal rings. Although Lu is similar in structure to other carotenoids, its relative orientation in
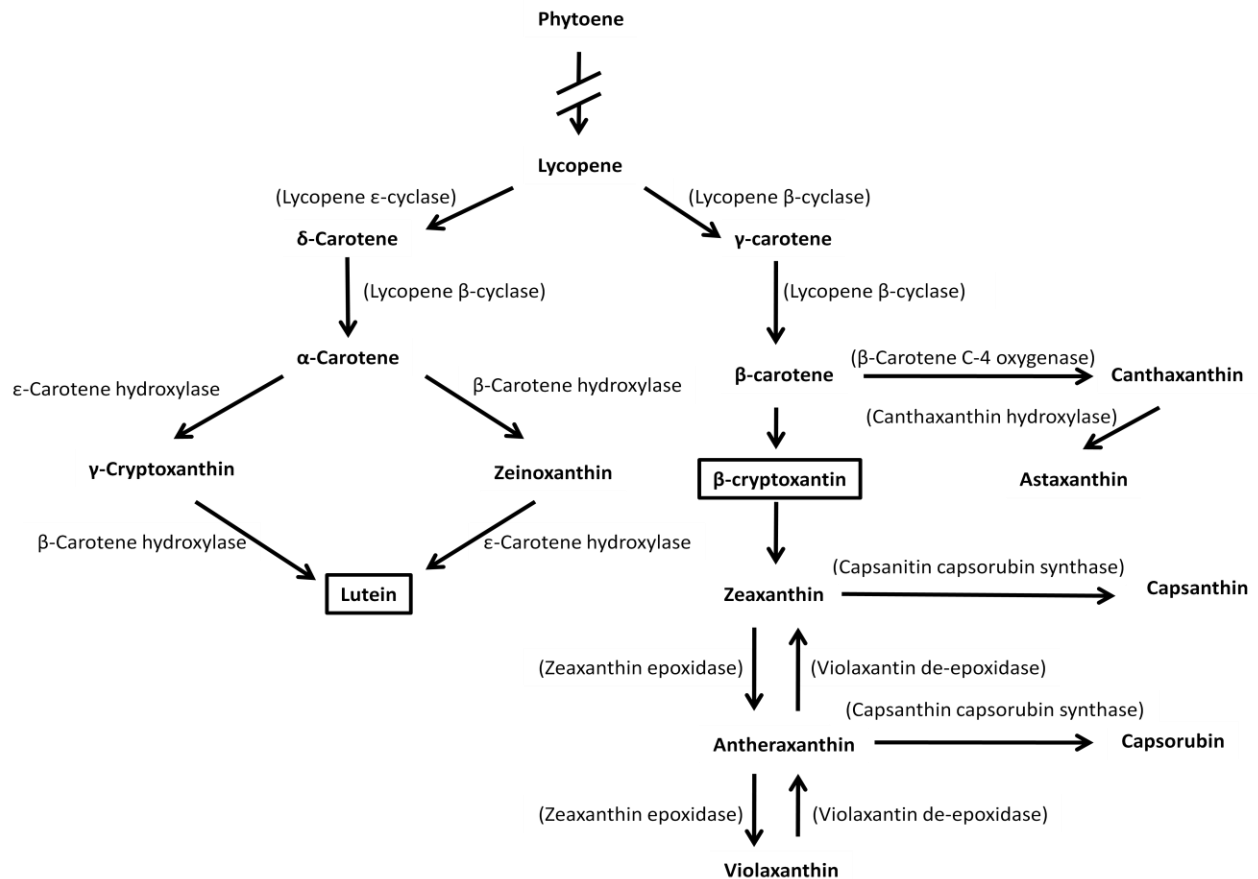
Phytoene

Lycopene

(Lycopene ε-cyclase) → δ-Carotene

(Lycopene β-cyclase) → γ-carotene

δ-Carotene → (Lycopene β-cyclase) → α-Carotene

γ-carotene → (Lycopene β-cyclase) → β-carotene

β-carotene → (β-Carotene C-4 oxygenase) → Canthaxanthin

α-Carotene → ε-Carotene hydroxylase → γ-Cryptoxanthin

α-Carotene → β-Carotene hydroxylase → Zeinoxanthin

β-carotene → β-cryptoxantin

(Canthaxanthin hydroxylase) → Astaxanthin

γ-Cryptoxanthin → β-Carotene hydroxylase → Lutein

Zeinoxanthin → ε-Carotene hydroxylase → Lutein

β-cryptoxantin → Zeaxanthin

Zeaxanthin → (Capsanitin capsorubin synthase) → Capsanthin

Zeaxanthin ⇄ Antheraxanthin (Zeaxanthin epoxidase) / (Violaxantin de-epoxidase)

Antheraxanthin → (Capsanthin capsorubin synthase) → Capsorubin

Antheraxanthin ⇄ Violaxanthin (Zeaxanthin epoxidase) / (Violaxantin de-epoxidase)

Figure 2. Biosynthesis of carotenoids by plants and algae (Bucsi and Poor 1998)

biological membranes influences cellular events at the incorporation site (Goodwin 1984). The hydroxyl rings in Lu also allow for a higher antioxidant activity than other carotenoids. βCr possesses a polar hydroxyl group at one of its two terminal rings. Unlike Lu, however, βCr is a provitamin A carotenoid; it can be cleaved in cells that produce oxygenase enzymes, such as intestinal cells, to form retinoid as well as apocarotenoids and aporetinoids. Lu and βCr can be protein-bound in plant leaves, esterified in fruits, and accumulate in lipophilic tissues in humans (Kaplan et al. 1990).
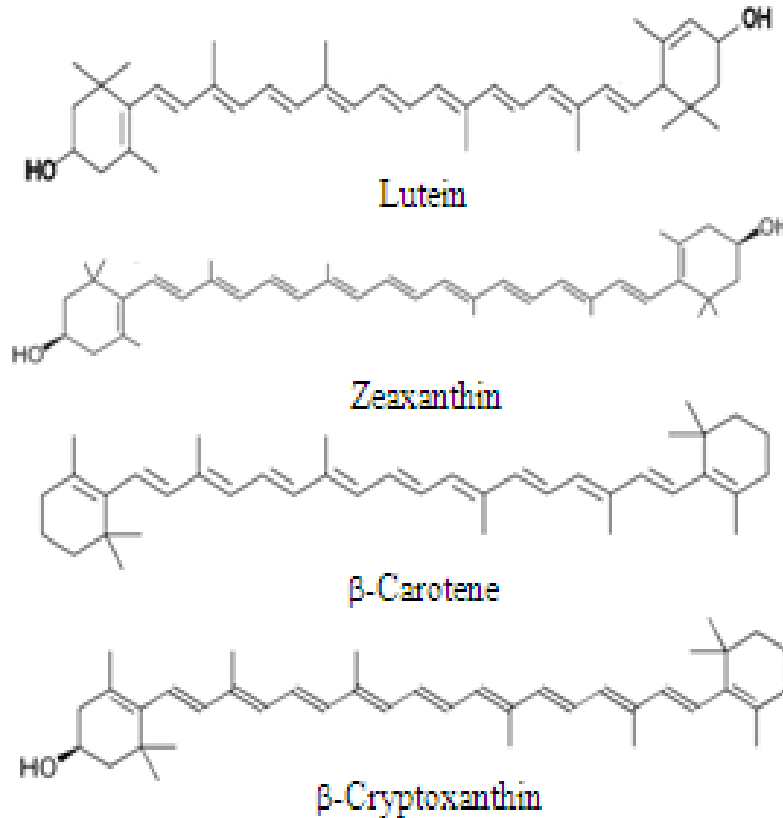
Figure 3. Chemical structures of lutein, zeaxanthin, β-carotene, and β-cryptoxanthin

7.2. Absorption

In food, Lu and βCr are incorporated into a tightly-bound matrix. Because of the highly

lipophilic nature of Lu and βCr, dietary lipid is an important factor in their absorption. The lipid

is emulsified by bile salts and integrated into micelles. From there, the lipids together with Lu

and βCr are taken up by chylomicrons and transported to the blood stream via the lymphatic

duct. In the blood, chylomicrons undergo lipolysis and the resulting chylomicron remnants are

then taken up by the liver and incorporated into high-density lipoproteins (HDL), low-density

lipoprotein (LDL), and very low-density lipoprotein (VLDL) before being transported to other

organs (Clevidence and Bieri 1993). The difference in polarity of individual carotenoids is thought to be highly associated with differences in metabolism. Lu and βCr are readily absorbed in humans. In fact, concentrations of Lu and βCr in human plasma and tissues are generally higher than other carotenoids.

7.3. Biological activity

Several studies have shown the biological activity of both Lu and βCr that make them useful and unique in downregulating factors involved in the progression of OA and RA. Lu has been shown to prevent systemic inflammation in response to lipopolysaccaride (LPS) and to modulate immune and inflammatory responses by regulating redox potentials (Jin et al. 2006, Koutos et al. 2006). In OA and RA, upregulation of NFκB results in increased production of pro-inflammatory cytokines and mediators, along with collagen degrading proteinases. Kim et al. (2008) recently showed that Lu is effective in reducing intracellular accumulation of H_2O_2 in murine macrophages, resulting in downregulation of NFκB. The protective effects of Lu on arthritic chondrocytes are currently unknown. βCr has been found to be associated with a reduced risk of developing inflammatory polyarthritis (IP) (Pattison et al. 2005). Even though the cellular mechanism in chondrocytes is not known, it is likely that βCr is involved in downregulating NFκB activation through its potent antioxidant activity. Therefore, Lu and βCr may act as potent antioxidants by inhibiting cellular damage to chondrocytes; the latter can lead to the production of pro-inflammatory cytokines and mediators, and proteinases responsible for cartilage degradation.

RESEARCH OBJECTIVES AND HYPOTHESES

In summary, OA and RA are both characterized by articular cartilage degeneration. Induction of

chondrocytes by IL-1β leads to increased cellular generation of ROS. Increased ROS

concentrations result in the upregulation of NFκB, leading to the production of matrix-degrading

proteinases, inflammatory cytokines, and mediators. Several studies have shown the antioxidant

activity of Lu and βCr that make them useful and unique in downregulating factors involved in

the progression of OA and RA. The objective of this study is to assess the protective effect of the

carotenoids, Lu and βCr, on NFκB activation, inflammatory response, and proteinase production

in SW-1353 human chondrosarcoma cells induced with IL-1β. We hypothesize that Lu and βCr

will reduce inflammatory response and proteinase production by downregulating NFκB.

MATERIALS AND METHODS

Cell Culture Conditions

Human SW-1353 chondrosarcoma cells (American Type Culture Collection, Rockville, MD, USA) were cultured in complete medium containing high glucose Dulbecco's modified Eagle's medium (DMEM; pH 7.3) supplemented with 100 units/mL penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B (Sigma Aldrich, St. Louis, MO, USA), and 10% newborn calf serum (NCS, HyClone, Logan, UT, USA). Cells were cultured to confluence in T-75 cm^2 flasks (Corning, Lowell, MA, USA) at 37°C in a humidified 5% CO_2 atmosphere. All experiments were conducted using cells within 2 passage numbers. At confluence, medium was aspirated from the flask and the cell layer rinsed three times with DMEM. After rinsing, cells were detached with 2 mL of trypsin-EDTA (0.25% trypsin, 0.05% EDTA in Hank's balanced salt solution without Mg^{2+} and Ca^{2+}) (Sigma Aldrich, St. Louis, MO, USA), transferred to 15 ml centrifuge tubes (Biologix, Lenexa, KS, USA) and centrifuged at 300 ×g for 8 min at 4°C. Cells were washed once with DMEM and resuspended in 1 mL complete medium.

Cell Sizing

Cell counts were acquired by diluting 10 µL of the cell suspension in a 1.5 mL microfuge tube (ISC BioExpress, Kaysville, UT, USA) containing 40 µL DMEM and adding 50 µL Trypan blue (0.4 % in PBS, pH 7.3, Sigma Aldrich, St. Louis, MO, USA). After gently mixing, the cell suspension was loaded onto a hemocytometer (Baxter Diagnostics Inc, McGaw Park, IL) and counted. The optimal count settings (f L) for this cell type on a particle counter (Z1 Coulter Particle Counter, Beckman Coulter, Fullerton, CA, USA) was then determined using the plateau method. Briefly, cell suspension was diluted 1:1000 in diluent (Isoton II Diluent, Beckman

Coulter, Fullerton, CA) in duplicate and gently mixed. Cell threshold was measured between 12 and 30 fL and recorded. Using hemocytometer counts as reference, an approximate threshold of 19 fL was determined to be optimal for accurate counting on the particle counter.

Cell Plating Density

In order to establish an appropriate plating density, cells were cultured to confluence in T-75 cm^2 flasks and passaged as before. After resuspension in DMEM and counting, cells were seeded at $4x10^5$, $2x10^5$, and $1x10^5$ cells/well in 6-well plates (Greiner Bio-One, Monroe, NC, USA). After a 48 hr incubation to allow for attachment and proliferation, the medium was removed, 3 mL of serum-free DMEM added, and cells incubated an additional 24 hr. A working stock of recombinant human IL-1β (R & D Systems, Minneapolis, MN USA) was prepared by diluting 10 μg IL-1β in 1 mL sterile phosphate buffer solution (PBS; 8 mmol/ L Na$_2$HPO, 2 mmol/ L NaH$_2$PO$_4$, and 0.15 mol/L NaCl, ph 7.3) containing 0.1% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO, USA). Immediately prior to IL-1β stimulation, the working solution was diluted 1:5000 in serum-free DMEM to a final concentration of 2 ng IL-1β/mL, the culture medium removed, and 2 mL of IL-1β working solution added. Cells were incubated for 48 hr, dissociated from the wells as previously described, and counted. The final cell density is illustrated in **Figure 4**. In order to measure differences in cell response to IL-1β, cells must be plated at a density that allows for adequate analysis of response variables at the end of incubation. Cells plated at too low of an initial density may not produce measureable amounts of the response variable, whereas cells plated at too high an initial density may plateau in their log growth.

Experiment 1: Lutein

Cells were passaged as previously described, washed once with DMEM, resuspended in complete medium, and counted. The first experiment was conducted to determine the protective effects lutein (Lu) on IL-1β-induced stress response in SW-1353 cells. Cells were plated at 2 x 10^5 cells/well (n = 6/treatment group) in 6-well plates with 3 ml complete medium and incubated for 48 hr.

The purity of Lu (Sigma Aldrich, St. Louis, MO, USA) was determined by spectrophotometric analysis (DU650B, Beckman Coulter, Fullerton, CA, USA). The Lu was dissolved in tetrahydrofuran (THF; Sigma Aldrich, St. Louis, MO, USA), optical density was determined at 450 nm, and purity calculated using an extinction coefficient ($E^{1\%}$) of 2469. Working stocks of Lu were prepared at concentrations of 0, 10^{-3}, 10^{-4} and 10^{-5} mol/L in THF. The final serum concentration in the DMEM culture medium was 5%. To prepare Lu working solutions 50 µL or the appropriate Lu stock were added to 2.5 mL FBS in dark sterile bottles with vigorous stirring. DMEM was added to a final volume of 50 mL. All procedures were conducted under yellow light. A working solution of IL-1β was prepared by diluting IL-1β 1:1000 in 5% FBS-complete medium to give a final IL-1β concentration of 10 ng/mL in the culture.

On the day of the study, medium was aspirated from cultured cells and 3 ml of 0, 0.01, 0.1, or 1.0 µmol Lu/L added to the respective wells. Cells were incubated in the presence of Lu for 24 hr. The medium was then replaced with 2 mL IL-1β solution (10ng/mL final concentration). Wells without Lu or IL-1β served as the negative control. Following a 24 hr incubation in the presence of IL-1β, the conditioned medium (CM) from each well was collected, aliquoted into five 1.5 mL microfuge tubes and stored at -80°C. Cells were dissociated from the

wells by the addition of 1 ml trypsin-EDTA and pipetted into 1.5 ml microfuge tubes containing

500 µL DMEM. The cells were washed once by centrifugation at 300 ×g for 5 min at 4°C. The

medium was discarded and the cell pellet resuspended in 500 µL of DMEM. Cells were counted

and the cell pellet snap-frozen and stored at -80°C until further analysis.

Experiment 2: **β-Cryptoxanthin**

The second experiment was conducted to determine the protective effects of βCr (Sigma

Aldrich, St. Louis, MO, USA) on IL-1β-induced stress response in SW-1353 cells. The

experimental design was similar to Experiment 1. The purity of βCr was determined by

spectrophotometric analysis at 456 nm in THF using an $E^{1\%}$ of 2399. Working stocks of βCr

were prepared in THF at 10^{-3}, 10^{-4} and 10^{-5} mol/L and 1X preparations of βCr diluted in culture

medium were prepared immediately prior to addition to cultures. The CM and cell pellets from

Experiment 1 and 2 were used in the following assays.

MMP-13

Total latent and active MMP-13 in the CM were analyzed using a commercially available

ELISA kit (SensoLyte MMP-13 ELISA Kit, AnaSpec, San Jose, CA, USA). In this assay, 100

µL of samples and standards were added to 96-well plates pre-coated with mouse anti-human

monoclonal antibody and incubated overnight at 4°C. The plates were washed, 100 µL of

biotinylated antibody added, and incubated for one hr on a shaker at RT. Horseradish peroxidase

(HRP)-streptavidin (100 µL) was then added and plates incubated for 45 min on a shaker at RT.

After washing twice, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was

added and allowed to react for 30 min on a shaker at RT in the dark. After the addition of the

stop solution (2 N H_2SO_4), absorbance was read at 450 nm. The lower limit of detection for

MMP-13 was ≤ 6 pg/ml.

PGE$_2$

PGE$_2$ in CM was analyzed by available ELISA (Parameter PGE$_2$, R & D Systems,

Minneapolis, MN, USA). In this assay, samples were diluted 3-fold. The sample (150 µL),

primary antibody (50 µL) solution and PGE$_2$ conjugate (50 µL) were then pipetted into 96-well

plates pre-coated with goat anti-mouse polyclonal antibody and incubated overnight at 4°C. The

plates were then washed, 200 µL of substrate solution added and allowed to react for 20 min at

RT in the dark. The reaction was stopped by adding 50 µL of the stop solution (2 N H_2SO_4) and

absorbance read at 450 nm. The lower limit of detection for PGE$_2$ was 8.5 pg/ml.

Cytokines

Pro-inflammatory cytokines IL-1α, IL-2, IFN-γ , IL-6, IL-8, TNF-α and anti-

inflammatory cytokines IL-4, IL-10 were analyzed in CM by chemiluminescent array format

ELISA (Quansys Q-Plex Cytokine Array, Logan, UT). This assay simultaneously measures

multiple cytokines. Briefly, 50 µL of the sample or standard were added to pre-coated multiplex

array 96-well plates and incubated for 1 hr on a plate shaker at RT. Following incubation, plates

were washed, 30 µL detection buffer added, and plates incubated for 1 hr on a shaker at RT.

After washing, 30 µL of HRP-streptavidin were added and plates incubated for an additional 15

min on a shaker at RT. The plates were washed and 40 µL substrate solution added. Digital

images (Canon EOS 40D, Canon, Irvine, CA, USA) were acquired using imaging capture

software (Digital Imaging Professional 3.3, Canon, Irvine, CA, USA). Data obtained was

analyzed using Quansys Q-View 2.5.2 software (Quansys Q-Plex Cytokine Array, Logan, UT). The lower limit of detection was 4.10, 1.10, ≤ 1.0, ≤ 1.0, 1.36, 2.11, 1.36, and ≤ 1.0 pg/mL for IL-1α, IL-2, IFN-γ, IL-6, IL-8, TNF-α, IL-4, and IL-10, respectively. Due to high concentrations, CM from the βCr cultures were diluted 10-fold and re-analyzed for IL-6 and IL-8. Lu samples also had high concentrations of IL-6 and IL-8 but were not re-analyzed.

NFκB (human p50)

Nuclear extract NFκB p50 was analyzed in the cell pellets using a commercially available ELISA kit [NFκB (human 50) Transcription Factor Assay, Cayman Chemical, Ann Arbor, MI, USA]. This assay requires purification of cellular nuclear extracts. Due to inadequate cell numbers, cell pellets from 3 samples in each treatment were pooled for analysis of NFκB. All buffer reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Buffers were prepared as follows. Briefly, the phosphatase inhibitor solution was prepared in 1X PBS (pH 7.4) by adding 0.02 mol/L NaF, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_2OV_4. This solution (500 μL) was then added to each pooled cell pellet. Samples were centrifuged at 300 $\times g$ for 5 min at 4°C. This step was repeated and then 500 μL ice-cold hypotonic buffer (0.2 mol/L HEPES, pH 7.5), 0.05 mol/L NaF, 1 μmol/L Na_2MoO_4, and 0.1 mmol/L EDTA (pH 7.5) added. The pellet was gently mixed, transferred to pre-chilled 1.5 ml microfuge tubes and incubated on ice for 15 min. Nonidet P-40 (50 μL, 10%) was added, samples gently mixed and centrifuged at 14,000 $\times g$ for 30 sec at 4°C. The supernatant containing the cytosolic fraction was transferred to a new 1.5 mL microfuge tube and stored at -80°C until further analysis. The extraction buffer [0.01 mol/L HEPES (pH 7.9), 0.1 μmol/L EDTA, 1.5 mmol/L $MgCl_2$, 0.42 mol/L NaCl, 20 mmol/L NaF, 1 mmol/L β-glycerophoshate, 10 mol/L

Na$_3$OV$_4$, and 25% glycerol (v,v)] was prepared prior to the assay. The protease inhibitors dithiothreitol (DTT; 0.5 mmol/L), phenylmethylsulphonyl fluoride (PMSF; 0.5 mmol/L), pepstatin A (1 µg/mL), leupeptin (1 µg/mL), and aprotinin (10 µg/ml) were prepared immediately prior to use. After adding 30 µL of the extraction buffer, samples were vortexed for 15 sec, incubated on ice with a rocker for 15 min on ice, vortexed and incubated an additional 15 min. Samples were then centrifuged at 14,000 ×g for 10 min at 4°C. The supernatant containing the nuclear fraction was transferred to a new 1.5 mL microfuge tube, snap frozen, and stored at -80°C until further analysis. Prior to the assay, fresh Transcription Factor Binding Assay buffer was prepared. The sample nuclear extract (10 µL/well) was added to a 96-well plate pre-coated with a specific double-stranded DNA (dsDNA) sequence containing the NF-κB response element, and incubated at 4°C overnight. After washing, 100 µL/well NF-κB (human p50) primary antibody was added and incubated for 1 hr at RT. The plates were washed, 100 µL/well goat anti-rabbit secondary antibody added and the plate incubated for 1 hr at RT. The developing solution (100 µL/well) was then added and color allowed to develop on a shaker for 45 min at RT. Absorbance was read at 450 nm. Percent change was calculated by dividing the optical density (OD) of each treatment sample by the, corresponding positive control (IL-1β only). Differences in p50, expressed as % change, were calculated by dividing positive control values by samples incubated with the carotenoids (Lu or βCr). These numbers were corrected for nuclear protein concentration, and normalized to % change from the positive control.

Nitrate/ Nitrite

Nitrate/nitrite (NO) in CM and cytosolic extracts of cell pellets were analyzed using a commercially available colorimetric assay kit (Nitrate/Nitrite Colorimetric Assay Kit [(LDH

Method), Cayman Chemical, Ann Arbor, MI, USA)]. In this assay, NADPH was used to enzymatically reduce nitrate to nitrite. Lactate dehydrogenase (LDH) was added to oxidize excess NADPH and Griess reagents were used to develop color. First, 30 µL sample, 10 µL NADPH solution (1 mmol/L), and 10 µL nitrate reductase mixture were added to 96-well plates and incubated for 40 min at RT. Following this incubation, 10 µL cofactor solution and 10 µL LDH solution were added and incubated for 20 min at RT. Then 50 µL Griess reagent R1 and 50 µL Griess reagent R2 were added to each well. Color was allowed to develop for 10 min and absorbance read at 540 nm. The lower limit of detection for NO was 2.5 µM.

Protein

Total protein in the nuclear extract and CM were analyzed using a colorimetric assay (Micro BCA Protein Assay Kit, Pierce, Rockford, IL, USA). Standard or samples diluted 1:30 in saline (0.9% NaCl) were pipetted into 96-well plates. The working reagent (150 µL) was added to each well and plates were incubated for 2 hr at 37°C. After incubation, the plates were cooled to RT and concentrations of protein determined at 562 nm. The lower limit of detection for protein was 0.5 µg/ml.

Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA) using the General Linear Models procedure, and treatment means were compared by LSD. Probability value of $P<0.05$ was considered statistically significant.

RESULTS

MMP-13. Cells incubated without IL-1β (negative control) produced basal levels of MMP-13, with average concentrations of 0.027 and 0.031 ng/1x10^6 cells for the Lu and βCr studies, respectively (data not shown). When IL-1β was added (positive control), SW-1353 cells produced higher amounts ($P<0.01$) of MMP-13; concentrations reached 4.96 (Lu study) and 4.21 (βCr study) ng/1x10^6 cells. Addition of Lu (**Figure 5**) to IL-1β-stimulated cultures significantly reduced MMP-13 concentrations. MMP-13 decreased from 4.96 to 3.26 ng/1x10^6 cells ($P<0.02$) in cultures pre-incubated with 1.0 μmol/L Lu. Concentrations of MMP-13 also was lower ($P<0.08$) in cultures treated with 0.01 μmol/L (4.00 ng MMP-13/1x10^6 cells), but not with 0.1 μmol/L Lu (4.02 ng MMP-13/1x10^6 cells). Conversely, βCr did not influence MMP-13 production, although MMP-13 concentrations (3.28 ng/1x10^6 cells) in the presence of 0.01 μmol/L βCr tended to be lower than cultures without βCr (**Figure 5**).

PGE$_2$. The concentration of PGE$_2$ in cells incubated without IL-1β averaged 249 and 116 pg/1x10^6 cells for the Lu and βCr studies, respectively (data not shown). In the presence of IL-1β PGE$_2$ concentrations (248 pg/1x10^6 cells) were unchanged in the Lu study but increased (165 pg/1x10^6 cells) in the βCr study. Production of PGE$_2$ generally decreased in a dose-dependent manner when cells were cultured in the presence of Lu; however, PGE$_2$ was significantly lower ($P<0.04$) only in cells treated with 1.0 μmol/L Lu (107 pg/1x10^6 cells). PGE$_2$ concentrations decreased significantly from 165 pg/1x10^6 cells in the control cultures to 65, 82, and 103 pg/1x10^6 cells in cultures containing 0.01 ($P<0.01$), 0.1 ($P<0.02$), and 1.0 ($P<0.07$) (**Figure 6**).

Pro-inflammatory cytokines (IL-1α, IL-2, IFN-γ, IL-6, IL-8, TNF-α). In the absence of IL-1β, concentrations of IL-1α averaged 4.8 and 3.0 pg/mg protein in Lu and βCr cultures,

respectively (data not shown). When IL-1β was added in the absence of carotenoids, IL-1α

increased ($P<0.01$) to 9.2 and 8.9 pg/mg protein in Lu and βCr cultures, respectively (**Figure 7**).

Pre-incubation with Lu did not significantly affect IL-1α production. Additions of βCr to cultures

prior to IL-1β stimulation generally decreased IL-1α concentrations. IL-1α decreased from 8.93

to 6.1 pg/mg protein ($P<0.05$) in cultures pre-incubated with 1.0 μmol/L βCr and to 6.4 pg/mg

protein ($P<0.08$) with 0.01 μmol/L βCr **(Figure 7)**.

Concentrations of IL-2 in the absence of IL-1β averaged 10.2 and 7.6 pg/mg protein for

Lu and βCr cultures, respectively. IL-1β increased ($P<0.05$) IL-2 concentrations to 18.9 and 26.7

pg/mg protein in Lu and βCr cultures, respectively. In the presence of 0.1 μmol/L Lu, IL-2

concentrations increased ($P<0.01$) to 35.0 pg/mg protein. Conversely, addition of βCr to cultures

prior to IL-1β stimulation generally resulted in a dose-dependent decrease in IL-2 concentrations.

IL-2 decreased ($P<0.04$) from 26.7 in control cultures to 14.8 pg/mg protein ($P<0.04$) in cultures

pre-incubated with 0.1 and 1.0 μmol/L βCr (**Figure 8**).

IL-1β stimulated ($P<0.05$) IFN-γ production by SW-1353 cells in both studies (not

shown). Production of IFN-γ generally increased in the presence of Lu; concentrations increased

significantly ($P<0.06$) with 1.0 μmol/L Lu. In contrast, IFN-γ concentrations were lower when

cells were incubated with 0.1 ($P<0.06$) and 1.0 ($P<0.07$) with βCr (**Figure 9**).

Cells incubated without IL-1β produced basal levels of IL-6 (7.0 pg/mg protein), IL-8

(28.5 pg/mg protein), and TNF-α (8.1 pg/mg protein) in Lu cultures. When IL-1β was added

concentrations of IL-6, IL-8, and TNF-α increased ($P<0.01$) significantly (data not shown).

Addition of 0.01, 0.1, or 1 μmol/L Lu or βCr to cultures had no effect on IL-6, IL-8, or TNF-α

production (data not shown).

Anti-inflammatory cytokines (IL-4 and IL-10). Cells cultured in the absence of IL-1β had basal concentrations of IL-4 (averaged 3.8 and 3.0 pg/mg protein, for Lu and βCr, respectively). When IL-1β was added to the cells, IL-4 increased ($P<0.01$) to 10.1 (Lu cultures) and 11.5 (βCr cultures) pg/mg protein. Addition of Lu to cultures prior to IL-1β stimulation significantly increased ($P<0.02$) IL-4 concentrations in cultures pre-incubated with 0.1 and 1.0 µmol/L Lu. Cultures pre-incubated with 0.01 µmol/L Lu also tended to produce more IL-4. Conversely, pre-incubation with βCr had no effect on IL-4 production **(Figure 10)**.

　　Concentrations of IL-10 in cultures not stimulated with IL-1β averaged 4.5 and 3.0 pg/mg protein for Lu and βCr cultures, respectively (data not shown). Addition of IL-1β stimulated ($P<0.01$) IL-10 production in both Lu and βCr cultures (**Figure 11**). Lu had no significant effect on IL-10 production, although 1.0 µmol/L Lu tended ($P<0.1$) to decrease IL-10 concentrations (**Figure 11**). Addition of βCr to cultures prior to IL-1β stimulation generally decreased IL-10 concentrations, with 1.0 µmol/L βCr significantly ($P<0.02$) inhibiting IL-10 production. Cultures pre-incubated with 0.01 µmol/L βCr tended to have lower ($P<0.1$) IL-10 **(Figure 11)**.

Nitrate/Nitrite in CM and cytosolic extract . Nitrate/nitrite in both conditioned medium and cytosolic extracts were generally below detection limits for both the Lu and βCr studies (data not shown).

NFκB (p50) in nuclear extract. Addition of 0.01, 0.1 and 1 µmol/L Lu reduced p50 expression in response to IL-1β to 68, 61, and 84% of control cultures, respectively (**Figure 12**). Addition of 1 µmol/L βCr reduced p50 expression to 62 %. However, addition of 0.01 and 0.1 µmol/L βCr increased p50 expression to 120 and 110%, respectively **(Figure 12)**.

DISCUSSION

In this study, the carotenoids Lu and βCr were evaluated for their protective effects against IL-1β-induced inflammatory response, and subsequent proteinase production and NFκB (p50) expression in the human chondrosarcoma cell line, SW-1353. Preincubation with Lu and βCr resulted in a protection against IL-1β-induced inflammatory response by triggering a number of modifications in NFκB p50, MMP-13, PGE$_2$, IL-1α, IL-2, IFN-γ, IL-4, and IL-10 production.

The transcription factor NFκB is often referred to as the "master switch" of inflammatory response, and plays a critical role in OA and RA progression. NFκB is present in nearly all human cells, including chondrocytes and resting chondrocytes where it exists in the cytosol as an inactive form to an inhibitor, IκB. It can be activated by inflammatory stimuli such IL-1β, TNF-α, and lipopolysaccaride (LPS) (Mengsol et al. 2000, Kim et al. 2008), and this upregulation of NFκB leads to increased production of pro-inflammatory cytokines (IL-1α, IL-1β, IL-2, IFN-γ, IL-6, IL-8, and TNF-α), pro-inflammatory mediators (PGE$_2$ and NO), and type II collagen degrading proteinases (such as MMP-13). Similarly, several ROS such as NO and H$_2$O$_2$ have been shown to activate NFκB in chondrocytes (Li and Engelhardt 2006, Rosa et al. 2008). Following a stimulus, intracellular signals activate the IκB kinase (IKK) complex, in turn induces phosphorylation and degradation of IκB from NFκB. Activation of the NFκB pathways results in the nuclear appearance of p50 and p65 and the subsequent transcriptional induction of inflammation-associated genes that encode pro-inflammatory cytokines, iNOS, COX-2, and MMP-13 (Kim et al. 2008).

Preincubation of IL-1β-stimulated SW-1353 cells with the carotenoids Lu and βCr resulted in decreased expression of nuclear p50 present, in this study, all concentrations of Lu decreased p50 expression including a 39% reduction in cultures containing 0.1 µmol/L Lu. These

results support the ability of Lu to downregulate NFκB activation. Similar results have been reported using murine macrophage cell line RAW 264.7 stimulated with LPS. In a study by Kim et al. (2008), Lu at 10 μmol/L inhibited NFκB activation in LPS-induced RAW 264.7 cells. In this study, βCr also decreased concentrations of nuclear p50 by 38% in cells cultured preincubated with 1.0 μmol βCr. There was no similar decrease with 0.1 μmol/L βCr; however, a study by Yamaguchi and Uchiyama (2008) utilizing osteoclastic cells similarly showed the inability of 0.1 μmol/L βCr to downregulate NFκB activation. This indicates that βCr is required in higher concentrations to prevent p50 translocation into the nucleus.

The ability of antioxidants to down-regulate NF-κB activation is likely due to their potent scavenging of ROS (Hamalainen et al. 2007). In this study, intracellular ROS were likely generated by IL-1β binding. Pretreatment of human chondrocytes with carotenoids resulted in clearance of ROS and inhibition of ligands required for downstream NFκB activation. Li and Englelhardt (2006) demonstrated that slight changes in H_2O_2 concentrations in IL-1β-induced human breast cancer cells (MCF-7) led to Rac1-dependent ROS production through NADPH oxidase, resulting in NIK activation and, ultimately, downstream activation of NFκB. These results are supported by Kim et al. (2008) who proposed that macrophages stimulate LPS binding, triggering downstream activation of NADPH oxidase, NIK, and subsequently NFκB. Interestingly, NADPH oxidase generates superoxide extracellularly, which is then converted to H_2O_2 before crossing the cytosolic membrane to interact with NIK. Other oxidants such peroxinitrite (ONOO⁻), which form when the production of NO and O_2^- is elevated, have also been shown to modulate NFκB activation, though their involvement is still highly debated (Gloire et al. 2006).

In OA and RA cartilage, NFκB activation results in increased MMP-13 production, resulting in the loss of type II collagen, tensile properties, and structural integrity of the affected joint. Numerous studies have demonstrated the ability of IL-1β to induce MMP-13 production in chondrocytes (Radons et al. 2006, Wada et al. 2006). Gebauer et al. (2005) confirmed that SW-1353 cells and PHC were similar in producing increased amounts of MMP-13 in response to IL-1β stimulation. The present study shows that Lu, at a physiological concentration (1.0 μmol/L), can decrease IL-1β-induced production of MMP-13 in SW-1353 cells. In contrast to Lu, βCr did not affect MMP-13 production. Therefore, results from this study suggest that Lu can protect against type II collagen degradation by decreasing MMP-13 concentration.

PGE_2 is a major pro-inflammatory mediator in arthritic diseases, and has been reported to not only potentiate inflammation but also inhibit proteoglycan synthesis (Torzilli et al. 1996). NO contributes to the development of OA and RA by mediating catabolic responses mediated by pro-inflammatory cytokines such as IL-1β. Chondrocytes induced by IL-1β upregulate NFκB resulting in the production of PGE_2 and NO through COX-2 and iNOS activation, respectively (Otero and Goldring 2007). In this study, basal PGE_2 concentrations were not increased by IL-1β induction, although PGE_2 was present in measurable amounts. The percentage of FBS in our culture medium was decreased from 10% to 5%. This decrease in FBS may have triggered the response of PGE_2 production in cultures without IL-1β. Wada et al. (2006) reported that SW-1353 cells induced with 10 ng/mL IL-1β produced no measurable PGE_2, but this study agrees with Merluzzi et al. (1987) who showed production of PGE_2 by SW-1353 cells with or without IL-1. We showed that Lu and βCr decreased PGE_2 concentrations by IL-1β-induced SW-1353 cells. At 1.0 μmol/L, Lu significantly decreased PGE_2 concentrations while lower Lu concentrations (0.1 μmol/L) also tended to reduce PGE_2 concentrations. Both 0.1 and 0.01

μmol/L βCr effectively decreased PGE$_2$ concentrations. These results imply that Lu and βCr can possibly alleviate PGE$_2$-modulated inflammation and joint degeneration common in OA and RA. In this study, NO was virtually undetectable in the CM and cytosolic fractions of IL-1β-induced SW-1353 cells, suggesting that NO may not be involved in NFκB activation. Rosa et al. (2008) was able to detect iNOS in the cytosolic extracts of PHC induced with 20 ng/mL IL-1β. Therefore, the non-detectable amounts of NO in this study is likely due to an inability of SW-1353 cells to express or synthesis iNOS.

IL-1β can also induce chondrocytes to produce several other pro-inflammatory cytokines, including IL-1α, IL-2, IFN-γ, IL-6, IL-8 and TNF-α (Otero and Goldring 2007). The addition of Lu to IL-1β-induced SW-1353 cell cultures had a very limited affect on pro-inflammatory cytokine production in this experiment. At 0.1 μmol/L, Lu tended to increase IL-1α and IFN-γ concentrations and increased IL-2 significantly. This possible pro-inflammatory effect of Lu, especially with IL-2 production, may be due in part to its pro-oxidant activity. Numerous factors can influence the pro-oxidant activity of a carotenoid, including carotenoid concentration and cell redox status. It is conceivable that changes in redox-sensitive transcriptional factors such as NFκB led to changes in cellular production of IL-1α, IL-2, and IFN-γ (Burton and Ingold. 1984). However, the highest concentration of Lu (1.0 μmol/L) would also be expected to increase pro-inflammatory cytokine production in this scenario. Contrary to this, the highest concentration of βCr (1.0 μmol/L) significantly decreased IL-1α and IL-2 concentrations, along with a tendency to reduce IFN-γ concentrations. IL-1α, IL-2, and IFN-γ are generally classified as Th1-moduating cytokines because of their ability to induce immune responses that lead to autoimmune diseases such as RA. We showed that βCr, but not Lu, has strong anti-inflammatory properties, thereby decreasing the production of these pro-inflammatory cytokines in

chondrocytes. These results correlate with the absence of p50 in the nuclear fractions of cells cultured in the presence of 1.0 µmol/L βCr, suggesting that downregulation of NFκB led to decreased expression of the pro-inflammatory cytokines IL-1α, IL-2, and IFN-γ.

IL-1β upregulated the production of pro-inflammatory cytokines IL-6, IL-8, and TNF-α, in SW-1353 cells. However, βCr did not alter the expression in chondrocytes. Lu also had no effect on the production of TNF-α by IL-1β-induced SW-1353 cells. Lee et al. (2003) reported that the carotenoid astaxanthin significantly decreased TNF-α production in LPS-stimulated macrophages. However, macrophages are much more redox-sensitive than chondrocytes and therefore, direct comparison among these carotenoids is not possible. In addition, research utilizing transgenic and knockout mouse models suggest that IL-6, IL-8 and TNF-α are pivotal in driving acute, rather than chronic inflammation, (van den Berg 2005). Perhaps Lu and βCr are better suited to handle chronic inflammatory responses, as seen with OA and RA. Culturing cells with carotenoids for a longer period before induction with IL-1β may allow greater regulatory control of IL-6, IL-8, and TNF-α expression.

IL-4 and IL-10 are generally regarded as Th-2-modulatory cytokines and inhibit many of the catabolic processes induced by pro-inflammatory cytokines. IL-4 is essentially undetectable in arthritic tissue, suggesting that an imbalance in Th1/Th2-modulated cytokines drives disease progression (Joosten et al. 1999). It has also been shown that IL-4 can inhibit MMP production in cartilage explants (Lacraz et al. 1992). In this study, IL-1β-induced SW-1353 cells cultured in the presence of 0.1 and 1.0 µmol/L Lu significantly increased IL-4 production. Addition of 0.01 µmol/L Lu to cultures tended to increase IL-4 production, though not significantly, while βCr (1.0 µmol/L) tended to increase IL-4 concentrations. Joosten et al. (1999) demonstrated that IL-4 treatment of DBA/-1J/Bom mice stimulated with collagen-induced arthritis (CIA) resulted in

reduced collagen and bone destruction. Interestingly, even though IL-4 has tissue-protective

properties, it was unable to act as a potent anti-inflammatory cytokine. This is consistent in our

study in which increased production of IL-4 by IL-1β-induced SW-1353 cells cultured in the

presence of Lu correlates to decreased production of collagen degrading factors such as MMP-13

and PGE$_2$. Previous research indicates that IL-10 is chondroprotective by downregulating MMP

and pro-inflammatory cytokines such as IL-6 (Lacraz et al. 1995). In this study, Lu at 0.01

µmol/L tended to increase IL-10 production. Interestingly, IL-10 production was significantly

increased by cells cultured with 1.0 µmol/L βCr. Radons et al. (2006) reported that human

chondrosarcoma cells lack functional IL-10 receptors due to a defect in IL-10R1 surface

expression, leaving cells unresponsive to IL-10 induction. This explains the upregulation of IL-

10 we observed in cells cultured with βCr. Considering, in general, βCr decreased pro-

inflammatory cytokine production, the ability of SW-1353 cells to respond to IL-10 could result

in its downregulation by NFκB. However, Lu tended to upregulate IL-10 when NFκB was not

activated, suggesting that IL-10 production is dependent on which transcriptional pathway is

being activated.

In conclusion, the carotenoids Lu and βCr protect against degenerative factors

unregulated by IL-1β-induced SW-1353 cells, likely by scavenging ROS required for NFκB

activation. In general, downregulation of NFκB resulted in decreased production of cytokines,

mediators, and proteinases which are upregulated in OA and RA. However, a decrease in PGE$_2$

production by cells cultured with 0.1 and 0.01 µmol/L βCr resulted in no change in p50

concentrations, showing that other transcriptional pathways may be involved with βCr. This

study also demonstrated unique properties of both carotenoids. Chondrocytes cultured in the

presence of Lu tended to strongly downregulate factors involved with cartilage destruction

(MMP-13 and PGE$_2$) and upregulate factors (IL-4) involved in tissue-protection, at the expense of downregulating pro-inflammatory cytokines. Conversely, chondrocytes cultured in the presence of βCr tended to strongly downregulate factors associated with pro-inflammatory response (IL-1α, IL-2, and IFN-γ) and cartilage destruction (PGE$_2$), while failing to downregulate matrix-degrading proteinases (MMP-13). These findings offer new perspectives from therapeutic approaches for treating both the symptomatic and degenerative processes characteristic of OA and RA.

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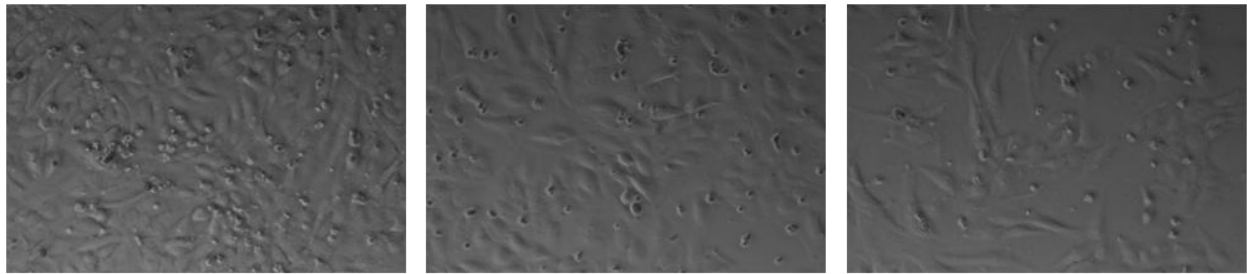
4x10⁵   **2x10⁵**   **1x10⁵**

Cell plating density/well

Figure 4. SW-1353 cells after 48 hr incubation in the presence of 2 ng/ml IL-1β. In 6-well plates, cells were seeded with 4×10^5 cells, wells were overly confluent with many cells floating/dead. However, in wells seeded at 2×10^5 cells, wells appeared confluent with few floating/dead cells. Wells seeded at 1×10^5 cells were under confluent.
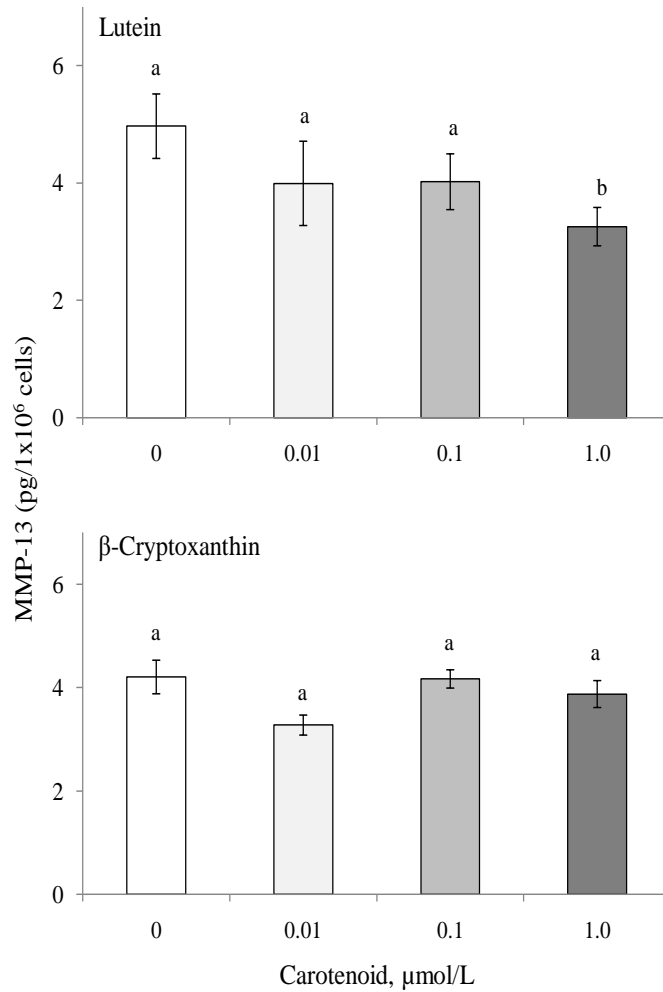
Figure 5. Production of MMP-13 (mean ± SEM) by SW-1353 cells incubated in the presence of

0, 0.01, 0.1, or 1.0 μmol/L /Lu or βCr/ and stimulated with 10 ng/mL IL-1β. Concentrations of

MMP-13 were expressed as per $1x10^6$ cells. Data were analyzed by ANOVA.

[a, b] Different letters above each bar denote significant differences between treatment groups
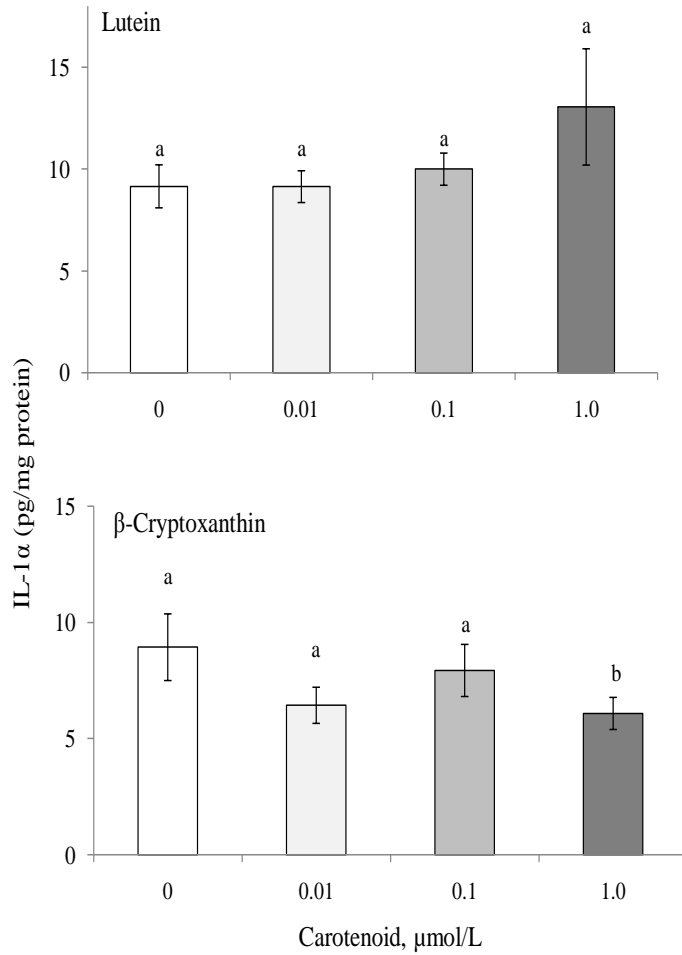
($P<0.05$).
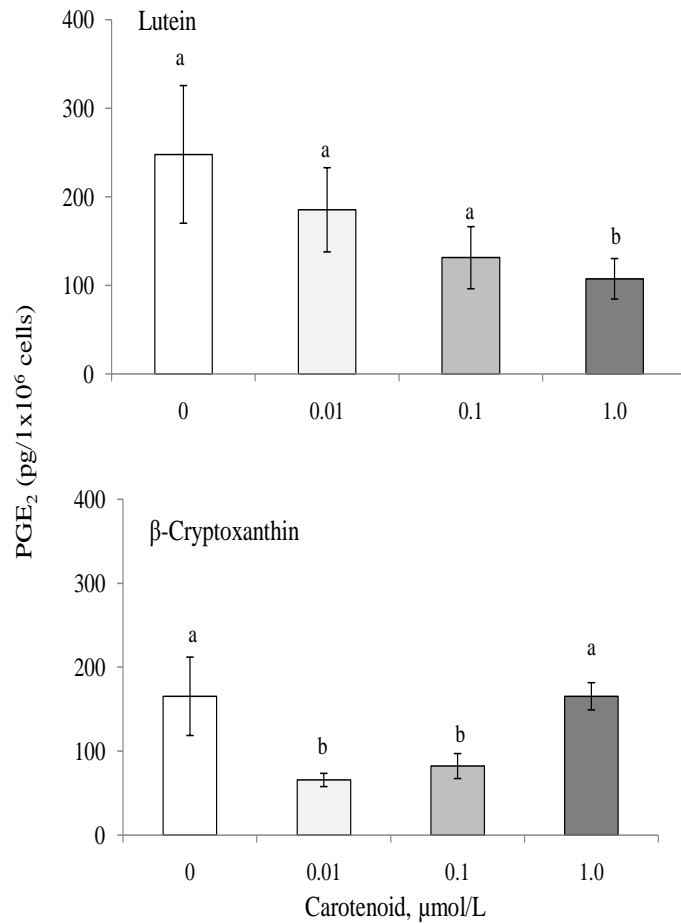
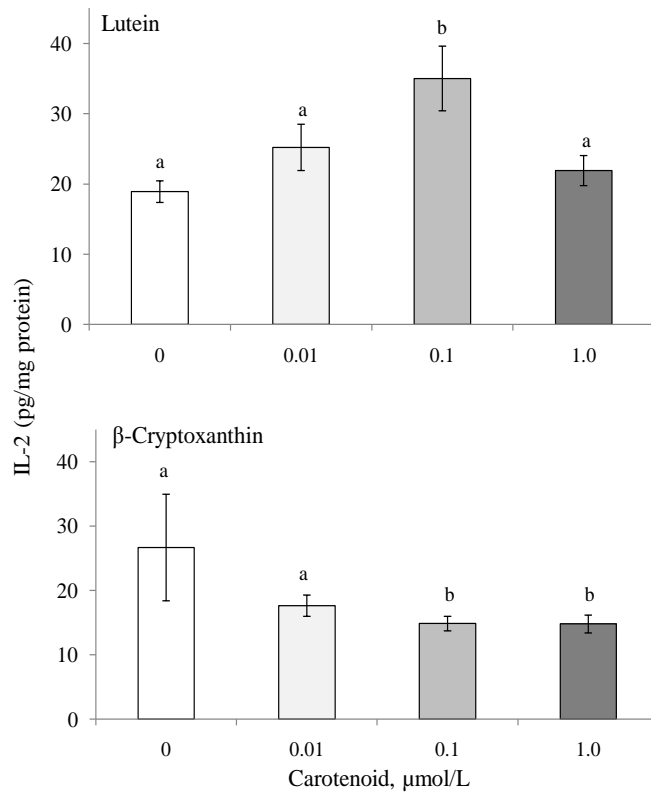**Figure 6.** Production of PGE_2 (mean $\pm$ SEM) by SW-1353 cells incubated in the presence of 0,

0.01, 0.1, or 1.0 µmol/L /Lu or βCr/ and stimulated with 10 ng/mL IL-1β. Concentrations of

PGE_2 were expressed as per 1×10^6 cells. Data were analyzed by ANOVA.

[a, b] Different letters above each bar denote significant difference between treatment groups

(p<0.05).

Figure 7. Production of IL-1α (mean ± SEM) by SW-1353 cells incubated in the presence of 0, 0.01, 0.1, or 1 µmol/L /Lu or βCr/ and stimulated with 10 ng/mL IL-1β. Concentrations of IL-1α were adjusted for protein concentrations. Data were analyzed by ANOVA.

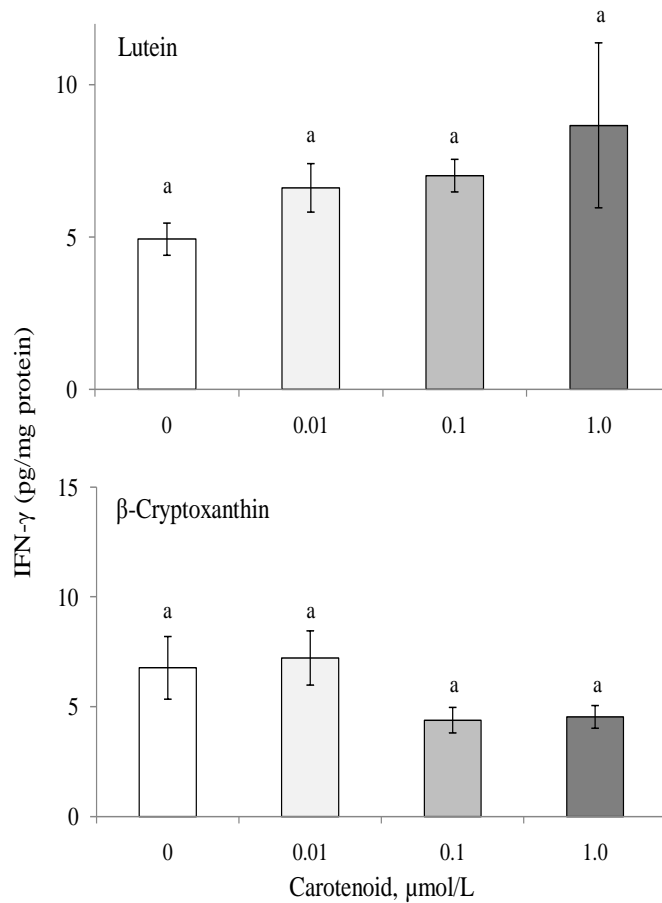[a, b] Different letters above each bar denote significant difference between treatment groups (p<0.05).

Figure 8. Production of IL-2 (mean ± SEM) by SW-1353 cells incubated in the presence of 0, 0.01, 0.1, or 1.0 µmol/L /Lu or βCr/ and stimulated with 10 ng/mL IL-1β. Concentrations of IL-2 were adjusted for protein concentrations. Data were analyzed by ANOVA.

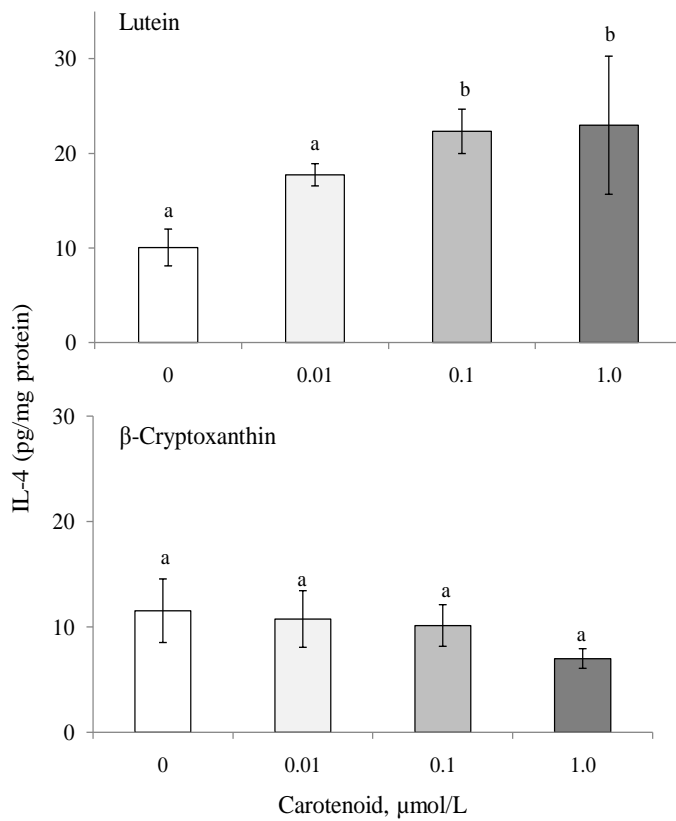[a, b] Different letters above each bar denote significant difference between treatment groups ($p<0.05$).

Figure 9. Production of IFN-γ (mean ± SEM) by SW-1353 cells incubated in the presence of 0,

0.01, 0.1, or 1.0 μmol/L /Lu or βCr/ and stimulated with 10 ng/mL IL-1β. Concentrations of

IFN-γ were adjusted for protein concentrations. Data were analyzed by ANOVA.

[a, b] Different letters above each bar denote significant difference between treatment groups

($p < 0.05$).

Figure 10. Production of IL-4 (mean ± SEM) by SW-1353 cells incubated in the presence of 0,

0.01, 0.1, or 1.0 µmol/L /Lu or βCr/ and stimulated with 10 ng/mL IL-1β. Concentrations of IL-4

were adjusted for protein concentrations. Data were analyzed by ANOVA.

[a, b] Different letters above each bar denote significant difference between treatment groups
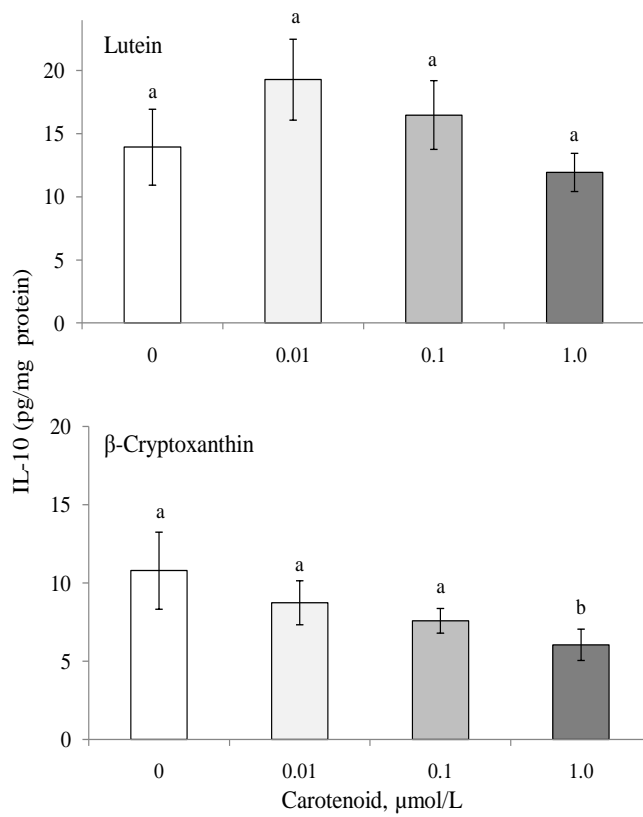
($p < 0.05$).

Figure 11. Production of IL-10 (mean ± SEM) by SW-1353 cells incubated in the presence of 0, 0.01, 0.1, or 1 μmol/L /Lu or βCr/ and stimulated with 10 ng/mL IL-1β. Concentrations of IL-10 were adjusted for protein concentrations. Data were analyzed by ANOVA.

[a, b] Different letters above each bar denote significant difference between treatment groups (p<0.05).
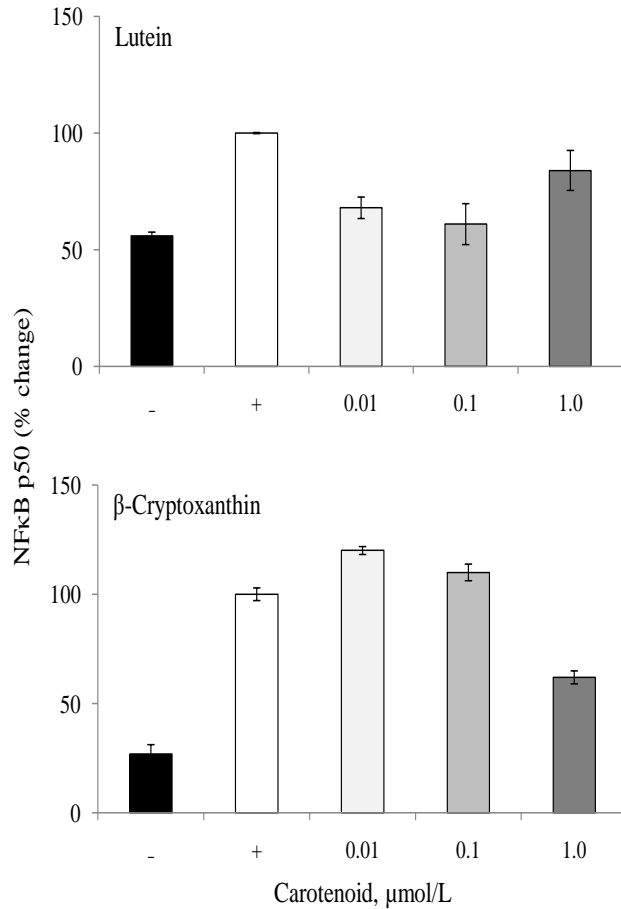
Figure 12. Production of NFκB p50 (mean ± SEM) in SW-1353 cells incubated in the presence of 0.01, 0.1, or 1 μmol/L /Lu or βCr/ and stimulated with 10 ng/mL IL-1β. In the negative (-) control wells were incubated with neither carotenoid or IL-1β, whereas the positive (+) control cells were incubated without carotenoid but with IL-1β. NFκB p50 responses (% change) were calculated by dividing positive controls by carotenoid (Lu or βcr) samples. These numbers were corrected for nuclear protein concentration, converted to percentages, and normalized to allow a positive control response of 100% change.

[a] Error bars represent SD