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Research Article

Astaxanthin Mediates Inflammation Biomarkers Associated with Arthritis in Human Chondrosarcoma Cells Induced with Interleukin-1β

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Abstract

Astaxanthin, a potent antioxidant, may downregulate inflammation-associated factors involved in arthritis. SW-1353 human chondrosarcoma cells were pre-incubated with 0, 0.01, 0.1, or 1.0 μmol/L astaxanthin for 48 h, and oxidative stress induced with 10 ng/mL IL-1β overnight. Astaxanthin (1.0 μmol/L) accumulated in SW-1353 cells in a time-dependent manner. IL-1β alone suppressed intracellular antioxidant activity, resulting in overproduction of reactive oxygen species (ROS), matrix metalloproteinase (MMP)-13, inflammatory cytokines and mediators. In contrast, pre-incubation with astaxanthin increased ($P < 0.05$) glutathione peroxidase activity and decreased ($P < 0.05$) ROS, MMP-13, IL-6, TNF-α and inflammatory mediators. Pre-treatment with astaxanthin also downregulated transcriptional activation of nuclear factor-κB and activator protein-1, which play critical roles in downstream production of MMP, inflammatory cytokines and mediators. Astaxanthin protects against degenerative factors upregulated by IL-1β, likely by scavenging ROS required for transcriptional activation.

Keywords: Arthritis; Astaxanthin; Chondrocyte; Inflammation

Abbreviations used: Activator protein-1 (AP-1); Culture supernatant (CS); Glutathione peroxidase (GPx); Leukotriene B_4 (LTB_4); Matrix metalloproteinase (MMP); Nuclear factor-κB (NF-κB); Osteoarthritis (OA); Prostaglandin E_2 (PGE_2); Reactive oxygen species (ROS); Rheumatoid arthritis (RA); Superoxide dismutase (SOD)

1. Introduction

Arthritis is the leading cause of disability among adults, with more than 17.4 million people experiencing activity limitations (Hootman et al., 2006). Osteoarthritis (OA) and rheumatoid arthritis (RA), the most common arthritic diseases, involve the progressive deterioration and loss of

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articular cartilage leading to debilitating impairment of joint function (Mazzetti et al., 2001). While the pathogenesis of OA and RA differ, these diseases are characterized by chondrocyte dysfunction leading to inflammation, activation of matrix-degrading proteinases and ultimately articular cartilage degeneration (Sangha, 2000).

IL-1β, a potent pro-inflammatory cytokine released by synovial cells *in vivo* during inflammation, plays a key role in cartilage degradation by stimulating the production of matrix metalloproteinases (MMP) in chondrocytes (Otero and Goldring, 2007). Similarly, human chondrocytes stimulated *in vitro* with IL-1β have been shown to increase production of MMP-1 and MMP-13 (Tetlow et al., 2001). IL-1β stimulation also results in the production of other pro-inflammatory cytokines and mediators, such as TNF-α, prostaglandin E_2 (PGE_2), and leukotriene B_4 (LTB_4) (Tetlow et al., 2001; Kojima et al., 2011). Further, IL-1β stimulation in chondrocytes leads to increased generation of cellular reactive oxygen species (ROS) and resulting oxidative stress from ROS overproduction. These highly reactive molecules cause cellular damage, including apoptosis, protein oxidation, DNA modification, and lipid peroxidation (Chew and Park, 2004). Additionally, ROS activate transcriptional messengers, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which up-regulate the production of matrix-degrading proteinases, inflammatory cytokines and mediators (Martin et al., 2005).

Under normal conditions, ROS concentrations are tightly controlled by endogenous antioxidant systems, including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. However, when these endogenous antioxidants are overwhelmed by the overproduction of ROS, oxidative stress occurs resulting in inflammation and cellular damage (Mazzetti et al., 2001), thereby requiring dietary antioxidants to help scavenge these harmful molecules. Therefore, dietary antioxidants may play a beneficial role in treating the pathogenesis and symptomatic manifestations seen in arthritis as they scavenge ROS and slow the progression of oxidative damage (Ahmed et al., 2002; Panico et al., 2005).

Individuals with RA have been shown to have lower plasma concentrations of carotenoids, including α-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene, as compared to those without RA (De Pablo et al., 2007). Astaxanthin, a keto-oxycarotenoid, has antioxidant activity that has been reported to be higher than that of β-carotene, α-carotene, and lutein (Naguib, 2000). Astaxanthin modulates oxidative stress and inflammatory mediators, and has been shown to be beneficial against multiple disease models (Fassett and Coombes, 2011). Astaxanthin inhibited inflammatory gene expression by suppressing NF-κB activation in lipopolysaccharide-stimulated RAW264.7 cells (Lee et al., 2003). Similarly, astaxanthin inhibited NF-κB activation in activated macrophages resulting in the downregulation of MMP and pro-inflammatory cytokine production (Kishimoto et al., 2010).

Although the protective effect astaxanthin on arthritic chondrocytes are not known, the ability of exogenous antioxidants to scavenge ROS and downregulate gene activation associated with the overproduction of inflammatory mediators and cytokines has been established. The objective of this study is to assess the protective effect of astaxanthin on inflammatory response in SW-1353

human chondrosarcoma cells induced with IL-1β. The SW-1353 cell line, derived from human chondrosarcomal cells, is a suitable model for examining pathological factors in OA and RA due to strong similarities to primary human chondrocytes with respect to catabolic effects after treatment with IL-1β, including activation of transcriptional regulators and the subsequent production of MMP, inflammatory cytokines and mediators (Gebauer et al., 2005; Lin et al., 2008).

2. Materials and Methods

2.1 Cell culture and carotenoid preparation

Human SW-1353 chondrosarcoma cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, and 10% newborn calf serum (HyClone, Logan, UT) at 37°C in a humidified 5% CO_2 atmosphere. Cell culture medium and all other chemicals were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise indicated. Adherent cells were detached with trypsin-EDTA (0.25% trypsin-0.05% EDTA in Hanks' balanced salt solution without Mg^{2+} and Ca^{2+}) and cell number enumerated using a Z1 Coulter Particle Counter (Beckman Coulter, Brea, CA). All experiments were conducted using cells within 2 passage numbers. Immediately prior to use, astaxanthin was first solubilized in tetrahydrofuran, gradually added to newborn calf serum while mixing, and the mixture slowly added to DMEM (final tetrahydrofuran concentration was 0.1%). The use of tetrahydrofuran is an acceptable medium for dissolving carotenoids in cell culture (Bertram et al., 1991).

2.2 Carotenoid kinetic uptake study

Cells were suspended in medium containing 1.0 μmol/L astaxanthin, plated at 2×10^6 cells/well in 6-well plates and incubated for 0, 3, 6, 12, or 24 h (n = 6) to study astaxanthin uptake by SW-1353 cells. At the end of each incubation period, cells were dissociated with trypsin-EDTA and lysed with 1 mL buffer containing 100 mM sucrose, 1 mM ethylene glycol tetraacetic acid, 20 mM 3-(N-morpholino)propanesulfonic acid (pH 7.4), and 0.1% bovine serum albumin. The hydrophobic fraction containing the carotenoid was sequentially extracted with 3 mL acetone containing 0.1% butylated hydroxytoluene followed by 3 mL hexane:ethyl acetate (1:1; v:v). After centrifugation the organic layer was collected, dried under nitrogen gas, and the residue dissolved in a mixture of hexane:acetone (82:18; v:v); the mobile phase used in the reverse phase HPLC separation (Alliance 2690, Waters, Milford, MA). Samples were eluted through a 3 μm silica column (150 x 4.6 mm, Luna, Phenomenex, Torrance, CA) and absorbance monitored at 474 nm (Photodiode Array Detector 996, Waters, Milford, MA).

2.3 Inflammatory biomarker assays

Cells were suspended in treatment medium supplemented with 0, 0.01, 0.1, or 1.0 μmol/L astaxanthin (n = 6) and plated at 5×10^5 cells/well in 6-well plates. After culturing 48 h, oxidative stress was induced with 10 ng IL-1β/mL culture medium and the cells incubated an additional 18 h. Negative control cultures received neither astaxanthin nor IL-1β, while positive control cultures only received the IL-1β. The resultant culture supernatant (CS) and cell pellet were collected and stored at -80°C until further analysis. Total latent and active MMP-13 in CS was analyzed by

sandwich ELISA (SensoLyte MMP-13, AnaSpec, Freemont, CA). The lower limit of detection for MMP-13 was ≤ 6 pg/mL. PGE_2 and LTB_4 were analyzed in CS by ELISA (Parameter PGE_2, Parameter LTB_4, R&D Systems, Minneapolis, MN), and the lower limit of detection was 30.9 and 27.6 pg/mL for PGE_2 and LTB_4, respectively. Pro-inflammatory cytokines IL-1α, IL-2, IL-6, IL-8, IFN-γ, and TNF-α were analyzed in CS by chemiluminescent array ELISA (Quansys Q-Plex Cytokine Array, Logan, UT) and data were analyzed using Quansys Q-View 2.5.2 software; the lower limit of detection was ≤ 1.0, 2.76, 3.81, ≤ 1.0, ≤ 1.0, and ≤ 1.0 pg/mL, respectively. In addition, IL-17 was analyzed in CS by ELISA (Human IL-17 Quantikine ELISA Kit, R&D Systems, Minneapolis, MN); the lower limit of detection for this assay was ≤ 15 pg/mL.

2.4 Antioxidant activity

Cell pellets were analyzed for GPx and SOD activity. Cell extracts were prepared by solubilizing the cell pellets in sonication buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 1 mM ethylene glycol tetraacetic acid, 210 mM mannitol, 70 mM sucrose) and sonicated 5 times with 2 sec pulses (Branson Sonifier, Danbury, CT) at 70% maximum setting. Cellular GPx activity was measured using a colorimetric assay (BIOXYTECH cGPx-340, OxisResearch, Foster City, CA) monitoring change in absorbance at 340 nm (μQuant, BioTek Instruments, Winooski, VT); the detection limit was 5.6 mU/mL GPx activity. Cellular SOD activity was also measured with a colorimetric assay, with a detection limit of 0.025 U/mL SOD (Superoxide Dismutase Assay, Cayman Chemical, Ann Arbor, MI).

2.5 Transcription factor analysis

The cell pellets were analyzed to determine NF-κB activity by ELISA (NF-κB human p50 Transcription Factor Assay, Cayman Chemical, Ann Arbor, MI). Nuclear extracts were prepared according to manufacturer directions with one modification: nuclear pellets were resuspended in 30 μL of extraction buffer. Protein concentrations in nuclear extracts were determined using a colorimetric assay (Micro BCA Protein Assay Kit, Pierce, Rockford, IL). After standardizing NF-κB activity using nuclear protein concentrations, results were expressed as % response compared to the positive control. To further elucidate the mechanism of action, an additional set of nuclear extracts (n = 6) were prepared using an alternate method (Nuclear Extraction Kit, Panomics, Freemont, CA) for AP-1 binding activity. SW-1353 cells were cultured with 0 or 0.1 μmol/L astaxanthin for 48 h and then oxidative stress induced with 10 ng/mL IL-1β, as previously described. AP-1 binding activity in these extracts was determined using an indirect capture ELISA (AP-1 Transcription Factor Kit, Panomics, Freemont, CA). Results were expressed as % response compared to the positive control after adjusting for protein concentrations (DC Protein Assay, Bio-Rad, Hercules, CA).

2.6 ROS production

ROS production was determined using carboxy-H_2DCFDA, a fluorescent marker for ROS in live cells (Image-iT LIVE Green Reactive Oxygen Species Detection Kit, Molecular Probes, Eugene, OR). To determine acute ROS production in response to IL-1β induction, cells were plated at 5×10^4 cells/well in 96-well plates and cultured with 0 or 0.1 μmol/L astaxanthin (n = 6), as described previously. Cells were washed with Hanks' balanced salt solution, 25 μmol/L carboxy-H_2DCFDA

added, incubated for 30 min at 37°C then washed again with Hanks' balanced salt solution. Following the addition of 10 ng/mL IL-1β, fluorescence was measured (ex 495 nm/em 529 nm) every 30 min for 2 h in a fluorescent plate reader (FLx800, BioTek Instruments, Winooski, VT). An additional culture containing to IL-1β served as a negative control. Chronic ROS response was measured in a similar manner, following an incubation of cells with IL-1β for 18 h.

2.7 Statistical analysis

Data was analyzed by ANOVA using the General Linear Model procedure of SAS and treatment means were compared using protected LSD. Probability values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 Carotenoid uptake

Astaxanthin accumulated in a time-dependent manner in SW-1353 cells incubated with 1.0 μmol/L astaxanthin. There was significant uptake at 3 h, with concentrations reaching 60.5 ± 6.6 pmol/10^6 cells at 24 h (**Fig. 1**). Astaxanthin was undetectable in parallel cultures not supplemented with astaxanthin.

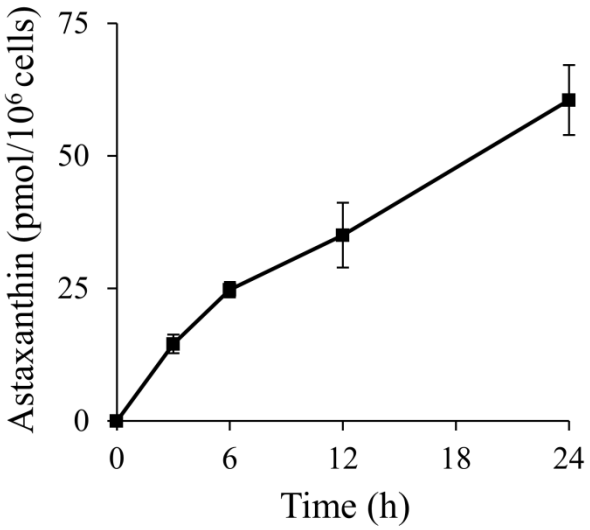

Fig 1. Accumulation of astaxanthin in SW-1353 cells incubated with 1.0 μmol/L astaxanthin for 0, 3, 6, 12, and 24 h. Values are means ± SEM.

3.2 Inflammation biomarkers

Following stress with IL-1β, SW-1353 cells showed increased ($P < 0.05$) production of MMP-13, PGE_2, and LTB_4 when compared to negative controls (**Table 1**). MMP-13 dramatically increased in response to IL-1β stimulation, while pre-incubation with astaxanthin decreased ($P < 0.05$) MMP-13 production in a dose-dependent manner. PGE_2 and LTB_4 production also decreased in a dose-

dependent manner in cells pre-incubated with astaxanthin. In the presence of 1.0 μmol/L astaxanthin, PGE_2 and LTB_4 production decreased ($P < 0.05$) to concentrations similar to those of the negative control cultures.

Table 1 Production of inflammatory biomarkers by SW-1353 cells pre-incubated with 0, 0.01, 0.1, and 1.0 μmol/L astaxanthin and subsequently stressed with 10 ng/mL IL-1β. Data are presented as means ± SEM. *Significantly different when compared to the IL-1β stimulated control, $P < 0.05$.

		Astaxanthin (μmol/L)			
		0	0.01	0.1	1.0
Biomarker (pg/μg protein)	No IL-1β	---------- IL-1β ----------			
MMP-13	0.6 ± 0.3*	83.6 ± 6.6	50.0 ± 7.8*	48.7 ± 6.0*	21.6 ± 2.1*
PGE_2	1.86 ± 0.24*	3.70 ± 0.73	2.95 ± 0.96	2.36 ± 0.19	1.70 ± 0.42*
LTB_4	1.81 ± 0.25*	4.77 ± 0.69	3.91 ± 0.77	3.93 ± 0.08	2.13 ± 0.55*

Table 2 Production of inflammatory cytokines by SW-1353 cells pre-incubated with 0, 0.01, 0.1, and 1.0 μmol/L astaxanthin and subsequently stressed with 10 ng/mL IL-1β. Data are presented as means ± SEM. *Significantly different when compared to the IL-1β stimulated control, $P < 0.05$.

		Astaxanthin (μmol/L)			
		0	0.01	0.1	1.0
Cytokine (pg/mL)	No IL-1β	---------- IL-1β ----------			
IL-1α	42.8 ± 0.9*	52.8 ± 0.6	59.9 ± 2.3*	86.3 ± 2.9*	60.5 ± 2.3*
IL-2	34.6 ± 0.5	37.4 ± 1.5	34.9 ± 2.0	38.7 ± 2.9	37.2 ± 2.6
IL-6	111.2 ± 2.5*	345.4 ± 4.1	210.8 ± 31.0*	220.0 ± 34.1*	164.7 ± 35.4*
IL-8	8.8 ± 0.7	10.0 ± 0.5	10.2 ± 0.9	9.3 ± 0.4	8.7 ± 0.5
IL-17	15.2 ± 1.9	19.9 ± 1.8	18.7 ± 3.9	18.1 ± 2.2	21.8 ± 3.5
IFN-γ	42.3 ± 9.8	50.8 ± 5.9	50.3 ± 1.6	51.3 ± 5.5	46.0 ± 2.3
TNF-α	57.3 ± 0.7*	67.7 ± 1.2	60.6 ± 1.7*	62.1 ± 1.7*	63.2 ± 1.5*

Incubation of SW-1353 cells with IL-1β in positive control cultures stimulated ($P < 0.05$) the production of the pro-inflammatory cytokines IL-1α, IL-6, and TNF-α (**Table 2**). The presence of astaxanthin in these cultures had mixed effects on these inflammatory cytokines. The production of IL-6 and TNF-α decreased ($P < 0.05$) with all concentrations of astaxanthin. Interestingly, pre-incubation with astaxanthin further increased ($P < 0.05$) IL-1α production. IL-2, IL-8, IL-17, and IFN-γ concentrations were not significantly different between control and astaxanthin treatment cultures (**Table 2**).

3.3 Antioxidant activity

GPx activity in SW-1353 cells was inhibited ($P < 0.05$) by IL-1β when compared to cells cultured without IL-1β (**Fig. 2**). Pre-incubation with astaxanthin increased ($P < 0.05$) GPx activity to levels similar to those of the negative control cultures. SOD activity was not significantly different between control and astaxanthin treatment cultures (overall mean ± SE, 162.5 ± 5.9 mU/10^6 cells).

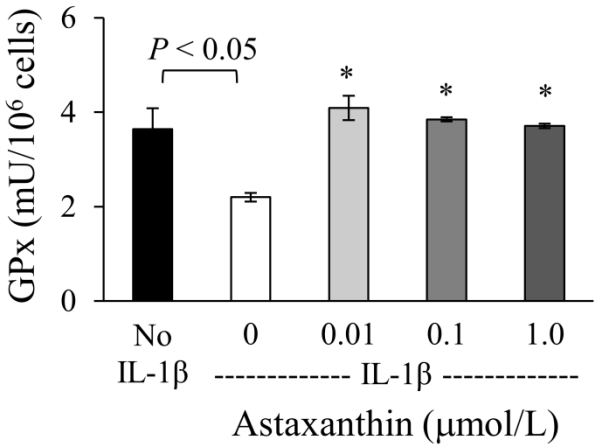

Fig 2. GPx activity (mean ± SEM) in SW-1353 cells pre-incubated with 0, 0.01, 0.1, and 1.0 μmol/L astaxanthin and subsequently stressed with 10 ng/mL IL-1β. An additional culture containing no IL-1β served as a negative control. *Significantly different when compared to the IL-1β stimulated control, $P < 0.05$.

3.4 Transcription factor assays

NF-κB p50 activity was stimulated ($P < 0.01$) in SW-1353 cells incubated in the presence of IL-1β when compared to cells cultured without IL-1β (**Fig. 3**). However, pre-incubation with 0.01 and 0.1 μmol/L astaxanthin decreased ($P < 0.05$) NF-κB activity up to 50%. Similarly, AP-1 activity also increased ($P < 0.01$) in the presence of IL-1β, while pre-incubation with 0.1 μmol/L astaxanthin inhibited ($P < 0.05$) AP-1 activity (**Fig. 4**).

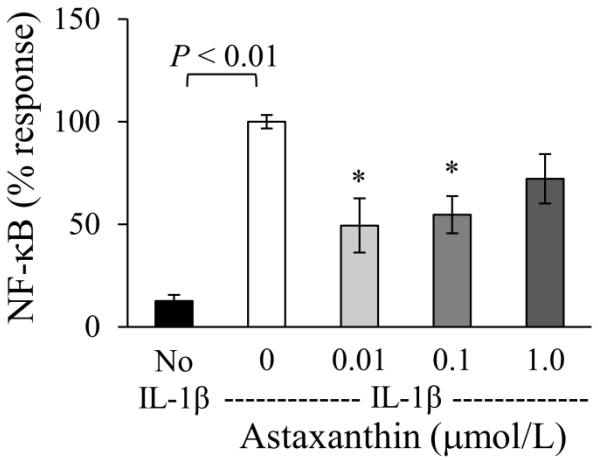

Fig 3. NF-κB activity (mean ± SEM) in SW-1353 cells pre-incubated with 0, 0.01, 0.1, and 1.0 μmol/L astaxanthin and subsequently stressed with 10 ng/mL IL-1β. An additional culture containing no IL-1β served as a negative control. *Significantly different when compared to the IL-1β stimulated control, $P < 0.05$.

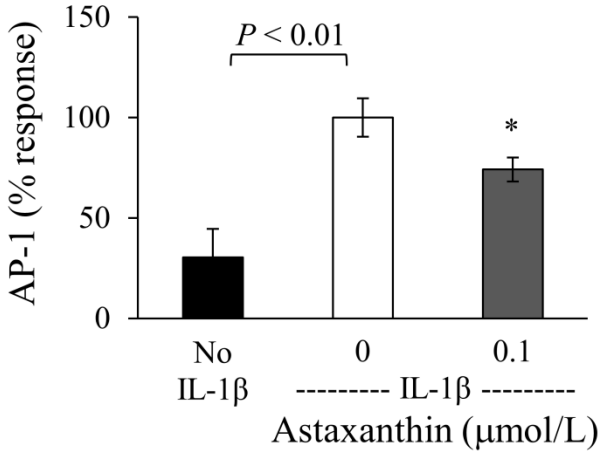

Fig 4. AP-1 activity (mean ± SEM) in SW-1353 cells pre-incubated with 0, 0.01, 0.1, and 1.0 μmol/L astaxanthin and subsequently stressed with 10 ng/mL IL-1β. An additional culture containing no IL-1β served as a negative control. *Significantly different when compared to the IL-1β stimulated control, $P < 0.05$.

3.5 ROS production

When compared to cells cultured without IL-1β, acute ROS production increased ($P < 0.05$) dramatically in IL-1β-stimulated cultures over the 120 min period studied (**Fig. 5**). Pre-incubation with 0.1 μmol/L astaxanthin decreased ($P < 0.05$) ROS production to levels similar to those observed in cultures not stimulated with IL-1β. ROS production also increased ($P < 0.01$) in positive control cultures after overnight stimulation with IL-1β. However, pre-incubation with 0.1 μmol/L

astaxanthin had no significant effect on ROS production after overnight stimulation with IL-1β (data not shown).

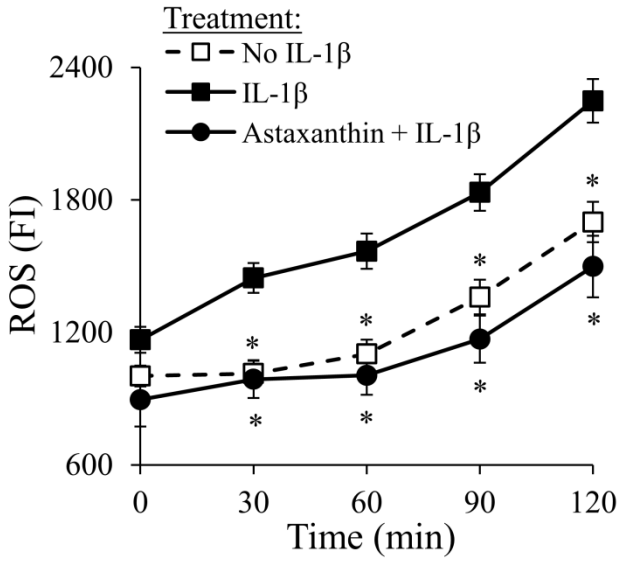

Fig 5. Acute ROS production (mean ± SEM) by SW-1353 cells pre-incubated with 0 or 0.1 μmol/L astaxanthin for 48 h and loaded with a 25 μmol/L caarboxy-H_2DCFDA working solution for 30 min at 37°C. Cells were stimulated with 10 ng/mL IL-1β and fluorescence intensity (FI) was measured every 30 min (ex 495 nm/em 529 nm). An additional culture containing no IL-1β served as a negative control. *Significantly different when compared to the IL-1β stimulated control, $P < 0.05$.

4. Discussion

The potent antioxidant and anti-inflammatory activity of astaxanthin is largely attributed to its transmembrane alignment in the lipid bilayer of cellular membranes. Astaxanthin has polar ionone rings at both ends of its non-polar conjugated carbon chain, allowing it to span the polar-nonpolar-polar lipid bilayer. In addition to preventing lipid oxidation, this alignment exposes the ionone rings to interact with ROS in the aqueous environment and provides proximity to cofactors such as vitamin C (Pashkow et al., 2008). We previously demonstrated that astaxanthin administrated orally is taken up in significant amounts by lymphocyte subcellular organelles of several species, thus showing specific uptake of the carotenoid by target cells (Park et al., 2010). Kinetic study of astaxanthin accumulation by SW-1353 human chondrosarcoma cells revealed that astaxanthin accumulated in a time-dependent manner with significant uptake after 3 h. Astaxanthin accumulation in SW-1353 cells results in a protection against IL-1β-induced inflammatory response by triggering a number of modifications in the production of inflammatory biomarkers.

Induction of oxidative stress with IL-1β resulted in suppressed GPx activity and the stimulation of several inflammatory biomarkers including MMP-13, PGE_2, LTB_4, IL-1α, IL-6, TNF-α, NF-κB, and AP-

1. Pre-incubation of SW-1353 cells with astaxanthin prior to stress induction restored GPx activity, indicating antioxidative action by astaxanthin. Interestingly, neither IL-1β stimulation nor astaxanthin treatment resulted in a similar response in SOD activity. SOD catalyzes superoxide dismutation to produce hydrogen peroxide that is then neutralized by GPx; it also serves to detoxify ROS to a limited capacity when GPx is inhibited or depleted (He et al., 2002). However, GPx and SOD are regulated through different signaling pathways (Mathy-Hartert et al., 2008); this likely explains the differences in SOD and GPx activity observed in this study.

Intracellular oxidative stress in response to IL-1β was further evidenced by the dramatic increase in ROS production in response to acute stimulation with IL-1β, and the continued elevation after 18 h IL-1β exposure. When endogenous antioxidant systems are overwhelmed by ROS, intracellular oxidative stress occurs and dietary antioxidants are required to restore optimal balance between oxidants and antioxidants. Pretreatment with astaxanthin decreased ROS production by SW-1353 cells in response to acute (120 min) stimulation with IL-1β. Therefore, stimulation with IL-1β suppressed antioxidant function, increased ROS accumulation and induced oxidative stress. However, pretreatment with astaxanthin alleviated this oxidative stress by restoring GPx activity and clearing intracellular ROS.

ROS act as second messengers activating transcription factors, such as NF-κB and AP-1, that upregulate the production of inflammatory cytokines and mediators. NF-κB plays a critical role in inflammation associated with the progression of arthritis and is often referred to as the "master switch" of inflammatory response. Activation of the NF-κB pathway results in the nuclear appearance of p50 and p65, and the subsequent transcriptional induction of genes associated with the production of inflammatory mediators and cytokines (Kim et al., 2008). Induction of SW-1353 cells with IL-1β increased NF-κB activity, indicating an upregulation of the NF-κB pathway. Pre-incubation with astaxanthin decreased NF-κB activity; ROS clearance by astaxanthin inhibits ligands required for downstream NF-κB activation (Hamalainen et al., 2007).

In addition to its NF-κB activity, IL-1β increases the activity of the serine-threonine kinases of the MAPK family. The p38 and JNK pathways have numerous effects, but one common target is the activation of AP-1. JNKs are termed stress-activated protein kinases since many of the JNK activators can be regarded as cellular stress (Lo et al., 1996). In the current study, AP-1 activity increased when SW-1353 cells were incubated with IL-1β, while pre-incubation with astaxanthin decreased AP-1 activity. The ability of antioxidants to downregulate NF-κB and AP-1 is likely due to their potent scavenging of ROS, inhibiting ligands required for downstream transcriptional activation and inflammatory gene expression.

Studies show that in rheumatic diseases, NF-κB is responsible for the transcription of inflammatory mediators such as PGE_2, while MMP are mainly activated through AP-1 (Martin et al., 2005; Lin et al., 2008). In OA and RA cartilage, MMP-13 production results in the loss of type II collagen, tensile properties, and structural integrity of the affected joint. Gebauer et al. (2005) confirmed that SW-1353 cells and primary human chondrocytes display similar MMP-13 production in response to IL-1β. Induction of SW-1353 human chondrosarcoma cells with IL-1β led to a drastic increase in the production of MMP-13, while pre-incubation with astaxanthin decreased MMP-13 production.

The activation of NF-κB and AP-1 also leads to the production of inflammatory mediators and cytokines. The induction of oxidative stress by IL-1β in SW-1353 cells resulted in the production of the pro-inflammatory mediators, PGE_2 and LTB_4, while pre-incubation with astaxanthin attenuated this response. PGE_2 potentiates inflammation and inhibits proteoglycan synthesis (Torzilli et al., 1996) while LTB_4 is involved in the induction of cytokines and MMP in the synovium (He et al., 2002). While some studies failed to identify the presence of 5-LOX or LTB_4 synthesis in chondrocytes (Wittenberg et al., 1993; Amat et al., 1998), it has been reported that the two major cofactors responsible for regulating leukotriene synthesis (FLAP and 5-LOX) are expressed in human OA chondrocytes and that chondrocytes are able to produce LTB_4 (Martel-Pelletier et al., 2004).

NF-κB activation is also associated with the production of inflammatory cytokines; however, IL-1β stimulation had a limited effect on inflammatory cytokine production in the present study. IL-1β induction of SW-1353 cells leads to the production of the pro-inflammatory cytokines IL-1α, IL-6, and TNF-α. On the other hand, pre-treatment with astaxanthin decreased IL-6 and TNF-α concentrations. While IL-1β plays a pivotal role in sustaining inflammation and cartilage erosion, TNF-α is also found in high concentrations in OA and RA tissues and may also play a role in the progression of these conditions. TNF-α functions to stimulate the production of pro-inflammatory mediators and catabolic proteinases in cartilage and may drive acute inflammation (Otero and Goldring, 2007). Along with IL-1β and TNF-α, IL-6 also plays an important role in inflammatory processes associated with arthritis. IL-6 has been shown to dysregulate enzymatic antioxidant defenses in chondrocytes, although to a lesser extent than IL-1β, leading to the accumulation of hydrogen peroxide in mitochondria and ultimately mitochondrial dysfunction (Mathy-Hartert et al., 2008). Interestingly, pre-incubation with astaxanthin further increased IL-1α concentrations. Numerous factors can influence pro-oxidant activity of a carotenoid including carotenoid concentration and cell redox status; β-carotene for example, has been shown to be a pro-oxidant at high doses (Burton and Ingold, 1984). Therefore, it is conceivable that changes in redox-sensitive transcriptional factors, such as NF-κB, leads to changes in cellular production of IL-1α.

IL-1α, IL-6, and TNF-α are generally classified as Th1-modulating cytokines due to their ability to induce an immune response (Schulze-Koops and Kalden, 2001). Recent evidence also suggests a role of Th17 cells in the pathogenesis of OA and RA, as Th17 cells are characterized by the production of the highly inflammatory IL-17. IL-17 has been shown to induce inflammation and joint destruction when administered *in vitro* and *in vivo* in animal models (Lundy et al., 2007). In chondrocytes, IL-17 induces the production of TNF-α, IL-1β, IL-6, MMP, and PGE_2. Further, IL-17 is upregulated by IL-1β and TNF-α thereby creating a positive feedback loop and self-perpetuating chronic inflammation (Lundy et al., 2007). However, there were no significant changes in IL-17 production following IL-1β stimulation or astaxanthin treatment in the current study.

In conclusion, astaxanthin accumulates in SW-1353 human chondrosarcoma cells and incubation with this carotenoid protects against inflammation and degenerative factors upregulated by IL-1β. The action of astaxanthin in ROS clearance helps to restore cellular antioxidant function and inhibits ligands required for downstream transcriptional activation and inflammatory gene

expression. In general, the downregulation of NF-κB and AP-1 resulted in the decreased production of matrix-degrading proteinases and inflammatory biomarkers associated with arthritis.

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