Astaxanthin Decreases Inflammatory Biomarkers Associated with Cardiovascular Disease in Human Umbilical Vein Endothelial Cells

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Abstract

Oxidative stress and inflammation are strongly linked to the development of cardiovascular disease (CVD). As a potent antioxidant, astaxanthin may downregulate inflammation-associated factors involved in endothelial dysfunction and CVD progression. We studied the possible protective effects of astaxanthin against endothelial dysfunction in human umbilical vein endothelial cells (HUVEC) induced with hydrogen peroxide (H₂O₂). Cells were pre-incubated with 0, 0.01, 0.1, or 1.0 μmol/L astaxanthin for 48 h, then oxidative stress induced with 100 μmol/L H₂O₂ overnight. Uptake kinetics of astaxanthin showed a time-dependent uptake of astaxanthin (1.0 μmol/L) by HUVEC over the 48 h incubation period. Oxidative stress induction with H₂O₂ in HUVEC decreased intracellular antioxidant activity and increased the production of inflammatory biomarkers and reactive oxygen species (ROS). In contrast, pre-incubation of HUVEC with astaxanthin prior to H₂O₂ stress increased (P < 0.05) superoxide dismutase activity and decreased ROS, prostaglandin E₂, leukotriene B₄, NO, IL-8, and IFN-γ production. Pre-treatment with astaxanthin also downregulated the transcriptional activation of NF-κB and activator protein-1, thereby inhibiting downstream production of inflammatory mediators and cytokines. Therefore, astaxanthin protects against factors initiated by H₂O₂ addition, likely by scavenging ROS required for transcriptional activation and inhibiting the production of inflammatory biomarkers involved in endothelial dysfunction and CVD.

Abbreviations: AP-1: activator protein-1; CVD: cardiovascular disease; CS: culture supernatant; EGM-2: endothelial growth medium-2; GPx: glutathione peroxidase; H₂O₂: hydrogen peroxide; HUVEC: human umbilical vein endothelial cell; LTB₄: leukotriene B₄; PGE₂: prostaglandin E₂; ROS: reactive oxygen species; SOD: superoxide dismutase; THF: tetrahydrofuran.

Keywords: Astaxanthin; HUVEC; Inflammation; Cardiovascular disease
1. Introduction

Cardiovascular disease (CVD) is the leading cause of death for both men and women in the United States, with more than 600,000 deaths attributed to CVD in 2006 (Lloyd-Jones et al., 2010). Certain conditions and lifestyle factors can greatly increase one's risk of developing CVD. While the relationships between these factors are complex, oxidative stress and inflammation are strongly linked to the development of CVD (Lee et al., 2011a). Despite this connection, there are few therapeutic interventions that successfully address this relationship between oxidative stress and CVD (Pashkow et al., 2008).

Oxidative stress results from the overproduction of reactive oxygen species (ROS). These highly reactive molecules cause cellular damage, including apoptosis, protein oxidation, DNA modification and lipid peroxidation (Chew and Park, 2004). The endothelial cells lining the blood vessels are very sensitive to injury caused by oxidative stress. Damage to endothelial cell structure and function contributes to blood vessel diseases, including atherosclerosis, thrombosis and vasculitis (Hafizah et al., 2010). Additionally, ROS activate transcriptional messengers, such as NF-κB and activator protein-1 (AP-1), both of which upregulate the production of inflammatory cytokines and mediators. Oxidative stress and inflammation resulting from the production of these inflammatory molecules are implicated in endothelial dysfunction and the subsequent development of CVD (Foncea et al., 2000).

ROS are natural byproducts of cellular respiration and are essential for cell signaling and homeostasis. Under normal conditions, ROS concentrations are tightly controlled by endogenous antioxidant systems, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. However, when endogenous antioxidants are overwhelmed by the overproduction of ROS, oxidative stress occurs resulting in inflammation and cellular damage (Lee et al., 2011a), thereby requiring dietary antioxidants to help scavenge these harmful molecules. Therefore, dietary antioxidants may play a beneficial role in treating CVD as they scavenge ROS and slow the progression of oxidative damage. Astaxanthin, a keto oxycarotenoid, is a potent antioxidant; its antioxidant activity 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 shown to be beneficial against multiple disease models (Fassett and Coombes, 2011). Astaxanthin inhibited NO production and inflammatory gene expression by suppressing NF-κB activation in lipopolysaccharide-stimulated RAW264.7 macrophage cell lines (Lee et al., 2003). Astaxanthin also suppressed serum NO and other inflammatory mediators in lipopolysaccharide-treated mice. Additionally, significant cardioprotection has been demonstrated with astaxanthin in multiple animal models of ischemia-reperfusion, with up to 70% protection from ischemic damage (Pashkow et al., 2008).

Endothelial cells play a crucial role in many vascular functions, including cell adhesion, inflammatory responses, regulation of permeability, and vasoactivity. Therefore, human umbilical vein endothelial cells (HUVEC) are commonly used to study vascular dysfunction (Hafizah et al., 2010). Further, hydrogen peroxide (H$_2$O$_2$) is a principal mediator of ROS-dependent signaling, and exposure of endothelial cells to H$_2$O$_2$ induces the activation of ROS-dependent signaling cascades (Eligini et al., 2009). Incubation of HUVEC with 100 μmol/L H$_2$O$_2$ has been shown to induce
oxidative stress, without inducing apoptosis (Lin et al., 2011), by inhibiting endogenous antioxidant activity and activating inflammatory signaling pathways (Hafizah et al., 2010).

In summary, the ability of exogenous antioxidants to scavenge ROS, such as H$_2$O$_2$ and NO, 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 HUVEC induced with H$_2$O$_2$.

2. Materials and Methods

2.1 Cell culture and carotenoid preparation
HUVEC (ATCC, Manassas, VA) were cultured in EGM-2 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 (THF), gradually added to newborn calf serum while mixing, and the mixture slowly added to EGM-2 (final THF concentration was 0.1%) while mixing. The use of THF is an acceptable medium for dissolving carotenoids in cell culture (Bertram et al., 1991).

2.2 Kinetic carotenoid uptake study
Cells were suspended in medium containing 1.0 µmol/L astaxanthin, plated at $5 \times 10^5$ cells/well in 6 well culture plates and incubated for 0, 6, 12, 24, or 48 h ($n = 6$) to study astaxanthin uptake by HUVEC. 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 $5 \times 10^5$ cells/well in 6 well plates. After culturing for 48 h, oxidative stress was induced with 100 µmol/L H$_2$O$_2$ and the cells incubated for an additional 18 h. Cultures containing no H$_2$O$_2$ served as a negative control. The resultant culture supernatant (CS) and cell pellet were collected and stored at -80°C until further analysis. Prostaglandin E$_2$ (PGE$_2$) and leukotriene B$_4$ (LTB$_4$) were analyzed in CS by ELISA (Parameter PGE$_2$, Parameter LTB$_4$, R&D Systems, Minneapolis, MN). The lower limits of detection were 30.9 and 27.6 pg/mL for PGE$_2$ and
LTB₄, respectively. NO was measured in CS by colorimetric assay with a lower limit of detection of 2.5 μmol/L (Nitrate/Nitrite Colorimetric Assay Kit, LDH method, Cayman Chemical, Ann Arbor, MI). Pro-inflammatory cytokines IL-1α, IL-1β, IL-2, IL-6, IL-8, IFN-γ, and TNF-α and anti-inflammatory cytokines IL-4 and IL-10 were analyzed in CS by chemiluminescent array ELISA (Quansys Q-Plex Cytokine Array, Logan, UT); data was analyzed using Quansys Q-View 2.5.2 software. The lower limit of detection was ≤ 1.0, 3.24, 2.76, 1.78, 3.81, ≤ 1.0, ≤ 1.0, and ≤ 1.0 pg/mL for IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ and TNF-α. 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-piperazinethanesulfonic 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 enzyme 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. HUVEC were cultured with 0 or 0.1 μmol/L astaxanthin for 48 h and then oxidative stress induced with 100 μmol/L H₂O₂, 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.

2.6 ROS production
ROS production was determined using carboxy-H₂DCFDA, 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 H₂O₂ induction, cells were plated at 5 x 10⁴ cells/well in 96-well plates and cultured with 0 or 0.1 μmol/L astaxanthin (n = 6), as described earlier. Cells were washed with Hanks’ balanced salt solution, 25 μmol/L carboxy-H₂DCFDA added, incubated for 30 min at 37°C then washed again with Hanks’ balanced salt solution. Following the addition of 100 μmol/L H₂O₂, fluorescence was measured every 30 min for 2 h (ex 495 nm/em 529 nm) in a fluorescent plate reader (FLx800, BioTek Instruments, Winooski, VT). An additional culture containing no H₂O₂ served as a negative control.
Chronic ROS response was measured in a similar manner, following an incubation of cells with H$_2$O$_2$ for 18 h.

2.7 Statistical analysis
Data were analyzed by ANOVA using the General Linear Model procedure 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 HUVEC incubated with 1.0 µmol/L astaxanthin. There was significant uptake at 6 h, with a maximum concentration of 125 ± 5 pmol/10$^6$ cells observed at 48 h (Fig. 1). Astaxanthin was undetectable in parallel cultures not supplemented with astaxanthin.

![Fig 1. Accumulation of astaxanthin in HUVEC incubated with 1.0 µmol/L astaxanthin for 0, 6, 12, 24, and 48 h. Values are means ± SEM.](image)

3.2 Inflammation biomarkers.
Following stress with H$_2$O$_2$, HUVEC showed increased ($P < 0.05$) production of PGE$_2$, LTB$_4$, and NO when compared to negative controls (Table 1). PGE$_2$ production was dramatically reduced ($P < 0.05$) in cultures incubated in 0.01, 0.1, as well as 1.0 µmol/L astaxanthin, compared to corresponding positive control cultures, with levels generally similar to those of the negative control cultures. Production of LTB$_4$ was reduced ($P < 0.05$) 40-65% when cells were incubated with astaxanthin compared to cultures without astaxanthin. NO production was similarly decreased ($P < 0.05$) approximately 25% in the presence of 0.1 and 1.0 µmol/L astaxanthin.
Table 1 Production of inflammatory biomarkers in HUVEC cultures pre-incubated in the presence of 0, 0.01, 0.1 and 1.0 µmol/L astaxanthin and subsequently stressed with 100 µmol/L H\(_2\)O\(_2\). Data are presented as means ± SEM. *Labeled means in a row represent differences compared to the H\(_2\)O\(_2\)-stimulated control, \(P<0.05\).

<table>
<thead>
<tr>
<th>Astaxanthin (µmol/L)</th>
<th>0</th>
<th>0.01</th>
<th>0.1</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>No H(_2)O(_2)</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>PGE(_2) (pg/µg protein)</td>
<td>2.01 ± 0.56*</td>
<td>20.28 ± 2.66</td>
<td>1.26 ± 0.44*</td>
<td>1.54 ± 0.50*</td>
</tr>
<tr>
<td>LTB(_4) (pg/mg protein)</td>
<td>119 ± 37*</td>
<td>955 ± 84</td>
<td>581 ± 122*</td>
<td>328 ± 32*</td>
</tr>
<tr>
<td>NO (nmol/µg protein)</td>
<td>0.49 ± 0.01*</td>
<td>2.30 ± 0.23</td>
<td>2.01 ± 0.14</td>
<td>1.65 ± 0.03*</td>
</tr>
</tbody>
</table>

Incubation of HUVEC with H\(_2\)O\(_2\) in positive control cultures stimulated \((P<0.05)\) the production of the pro-inflammatory cytokines IL-2, IL-6, IL-8, IL-17, IFN-\(\gamma\) and TNF-\(\alpha\) (Table 2). The presence of astaxanthin in these cultures had mixed effects on these inflammatory cytokines. At all concentrations of astaxanthin, IL-8 production decreased \((P<0.05)\), whereas only 0.1 µmol/L astaxanthin significantly inhibited \((P<0.05)\) IFN-\(\gamma\) production. Pre-incubation of HUVEC with astaxanthin had no effect on IL-2, IL-6 and TNF-\(\alpha\). Interestingly, pre-incubation with 0.01 µmol/L astaxanthin further increased \((P<0.05)\) IL-17 production. IL-1\(\alpha\) and IL-1\(\beta\) concentrations were not significantly different between controls and astaxanthin treatment cultures (overall means ± SE were 0.27 ± 0.06 and 0.71 ± 0.10 pg/µg protein, respectively).

Table 2 Production of cytokines in HUVEC cultures pre-incubated in the presence of 0, 0.01, 0.1 and 1.0 µmol/L astaxanthin and subsequently stressed with 100 µmol/L H\(_2\)O\(_2\). Data are presented as means ± SEM. *Labeled means in a row represent differences compared to the H\(_2\)O\(_2\)-stimulated control, \(P<0.05\).

<table>
<thead>
<tr>
<th>Cytokine (pg/µg protein)</th>
<th>No H(_2)O(_2)</th>
<th>0.01</th>
<th>0.1</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>0.29 ± 0.04*</td>
<td>1.52 ± 0.42</td>
<td>1.32 ± 0.38</td>
<td>0.84 ± 0.07</td>
</tr>
<tr>
<td>IL-6</td>
<td>15.1 ± 0.9*</td>
<td>39.3 ± 4.5</td>
<td>37.9 ± 2.7</td>
<td>34.4 ± 2.9</td>
</tr>
<tr>
<td>IL-8</td>
<td>29.2 ± 1.1*</td>
<td>136.4 ± 13.8</td>
<td>82.2 ± 7.0*</td>
<td>79.3 ± 5.0*</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.93 ± 0.07*</td>
<td>1.89 ± 0.21</td>
<td>6.43 ± 0.60*</td>
<td>3.88 ± 0.53</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>0.05 ± 0.02*</td>
<td>0.20 ± 0.04</td>
<td>0.13 ± 0.04</td>
<td>0.09 ± 0.04*</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>0.47 ± 0.12*</td>
<td>1.35 ± 0.32</td>
<td>1.15 ± 0.18</td>
<td>1.11 ± 0.25</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.15 ± 0.02*</td>
<td>0.45 ± 0.10</td>
<td>0.31 ± 0.06</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.13 ± 0.01*</td>
<td>0.51 ± 0.04</td>
<td>0.48 ± 0.04</td>
<td>0.50 ± 0.08</td>
</tr>
</tbody>
</table>
Incubation of HUVEC with H₂O₂ in positive control cultures also stimulated \( (P < 0.05) \) the production of the anti-inflammatory cytokines IL-4 and IL-10 (Table 2). However, astaxanthin had no effect on the production of these anti-inflammatory cytokines compared to the positive control.

3.3 Antioxidant activity.
SOD activity in HUVEC was inhibited \( (P < 0.01) \) by H₂O₂ when compared to cells cultured without H₂O₂; however, pre-incubation with astaxanthin increased \( (P < 0.05) \) SOD activity in a dose-dependent manner (Fig. 2). GPx activity also decreased \( (P < 0.01) \) in the presence of H₂O₂, with concentrations averaging 0.15 ± 0.01 and 0.01 ± 0.01 mU/µg protein in the negative and positive control cultures, respectively. Pre-incubation with astaxanthin had no effect on GPx activity (overall treatment mean ± SE, 0.01 ± 0.01 mU/µg protein).

Fig 2. SOD activity (mean ± SEM) in HUVEC pre-incubated with 0, 0.01, 0.1, or 1.0 µmol/L astaxanthin for 48 h and subsequently stressed with 100 µmol/L H₂O₂. An additional culture containing no H₂O₂ served as a negative control. *Significantly different from positive control \( (P < 0.05) \).

3.4 Transcription factor activation.
NF-κB p50 activity was stimulated \( (P < 0.01) \) in HUVEC incubated in the presence of H₂O₂ when compared to cells cultured without H₂O₂ (Fig. 3). However, pre-incubation with astaxanthin decreased \( (P < 0.05) \) nuclear p50 activity by 30-40%. Similarly, AP-1 activity also increased \( (P < 0.01) \) in the presence of H₂O₂ while pre-incubation with 0.1 µmol/L astaxanthin dramatically inhibited \( (P < 0.05) \) AP-1 activity (Fig. 4).
Fig 3. NF-κB p50 activity (mean ± SEM) by HUVEC pre-incubated with 0, 0.01, 0.1, or 1.0 µmol/L astaxanthin for 48 h and subsequently stressed with 100 µmol/L H₂O₂. An additional culture without H₂O₂ served as the negative control. Differences in p50 activity (expressed as percent response) were calculated by dividing the optical density (OD) of each treatment sample by the OD of the positive control. *Significantly different from positive control (P < 0.05).

Fig 4. AP-1 activity (mean ± SEM) by HUVEC cells pre-incubated with 0.1 µmol/L astaxanthin for 48 h and subsequently stressed with 100 µmol/L H₂O₂. An additional culture containing no H₂O₂ served as a negative control. Differences in AP-1 (expressed as percent response) were calculated by dividing the optical density (OD) of each treatment sample by the OD of the positive control. *Significantly different from positive control (P < 0.05).
3.5 ROS production.
While ROS production by HUVEC was very low in the absence of H$_2$O$_2$, acute ROS production increased ($P < 0.05$) dramatically and linearly in H$_2$O$_2$-stimulated cultures over the 120 min period studied (Fig. 5). Pre-incubation with 0.1 µmol/L astaxanthin decreased ($P < 0.05$) ROS production; however, ROS concentrations in the presence of astaxanthin were higher than observed in cultures not stimulated with H$_2$O$_2$. Similarly, ROS production increased ($P < 0.01$) in positive control cultures after overnight stimulation with H$_2$O$_2$. Pre-incubation with 0.1 µmol/L astaxanthin decreased ($P < 0.05$) ROS production by 44% as compared to the positive control (data not shown).

**Fig 5.** Acute ROS production (mean ± SEM) by HUVEC pre-incubated with 0.1 µmol/L astaxanthin for 48 h and loaded with a 25 µmol/L carboxy-H$_2$DCFDA working solution for 30 min at 37°C. Cells were stimulated with 100 µmol/L H$_2$O$_2$ and fluorescence intensity (FI) was measured every 30 min (excitation 495 nm/ emission 529 nm). An additional culture containing no H$_2$O$_2$ served as a negative control. *Significantly different from positive control ($P < 0.05$).

4. Discussion

The high antioxidant and anti-inflammatory activity of astaxanthin is largely attributed to its precise transmembrane alignment in the lipid bilayer of cellular membranes; the presence of polar ionone rings at both ends of the non-polar conjugated carbon chain allows the astaxanthin molecule to span the polar-nonpolar-polar lipid bilayer. In addition to preventing lipid-based oxidation, this alignment exposes the ionone rings to interact with ROS in the aqueous environment and likely provides proximity to cofactors, such as vitamin C (Pashkow et al., 2008). We previously demonstrated that astaxanthin administered orally is taken up in significant amounts by subcellular organelles of canine and feline lymphocytes, thus showing specific uptake of the carotenoid by target cells (Park et al., 2010). Kinetic study of astaxanthin accumulation by HUVEC showed significant, albeit low, uptake at 6 h, with maximal concentration observed at 48 h.
Astaxanthin accumulation in HUVEC protects these cells against H$_2$O$_2$-induced oxidative stress by triggering a number of modifications in the production of inflammatory biomarkers.

Oxidative stress was successfully induced in HUVEC cultures with 100 μmol/L H$_2$O$_2$, as evident from suppressed SOD and GPx activities, and the stimulation of several biomarkers including PGE$_2$, LTB$_4$, NO, IL-2, IL-6, IL-8, IL-17, IFN-γ, TNF-α, NF-κB, and AP-1. Pre-incubation of HUVEC with astaxanthin restored SOD activity, indicating antioxidative action by astaxanthin; but it had no significant effect on GPx activity suggesting different actions on these two endogenous antioxidants. Cytosolic GPx in endothelial cells has been shown to be inactivated by mediators of inflammatory response, including superoxide anion, hypochlorous acid, and NO (Moutet et al., 1998). Resveratrol increased both SOD and GPx activity in response to DMNQ-induced oxidative stress in HUVEC (Spanier et al., 2009).

Induction of oxidative stress in HUVEC is further evidenced by the dramatic increase in ROS production in response to an acute stimulation by H$_2$O$_2$, and the continued elevation after 18 h H$_2$O$_2$ exposure. Pretreatment with astaxanthin decreased ROS production in response to both acute and long term stimulation with H$_2$O$_2$. Resveratrol, epigallocatechin gallate, quercetin, and a combination of simvastatin and nifedipine also have been shown to reduce ROS production in HUVEC stimulated with H$_2$O$_2$ (Choi et al., 2005; Chen et al., 2010; Kao et al., 2010). Therefore, stimulation with H$_2$O$_2$ suppressed antioxidant function, increased ROS accumulation, and induced oxidative stress. However, pretreatment with astaxanthin alleviated this oxidative stress by restoring SOD 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 atherosclerosis and is often referred to as the “master switch” of inflammatory response. NF-κB exists in the cytosol in an inactive form bound to an inhibitor IκB, and following inflammatory stimuli, intracellular signals activate the IκB kinase complex, in turn inducing phosphorylation and degradation of IκB from NF-κB. 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). While several ROS play a role in the activation of NF-κB, H$_2$O$_2$ specifically modulates NF-κB by promoting the activation of the NIK/IKK pathway (Kim et al., 2008). HUVEC incubated in the presence of H$_2$O$_2$ showed an increase in nuclear p50 activity, indicating the upregulation of the NF-κB pathway. However, pretreatment with astaxanthin decreased nuclear p50 activity. Similarly, a purified extract of herbs commonly used for treatment of inflammatory diseases in Asia, also was shown to downregulate NF-κB activation in HUVEC stimulated with 10 ng/mL TNF-α (Mo et al., 2007).

In addition to its NF-κB activity, H$_2$O$_2$ 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). HUVEC incubated in the presence of H$_2$O$_2$ also showed an upregulation of AP-1; in contrast, pretreatment 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.

The activation of NF-κB and AP-1 leads to the production of inflammatory mediators and cytokines. Oxidative stress induction with H₂O₂ increased the production of PGE₂, LTB₄, and NO in HUVEC. However, pre-incubation with astaxanthin decreased the production of these inflammatory mediators. In addition to NO, the arachidonic acid metabolites (PGE₂ and LTB₄) are key markers of endothelial integrity. In fact, inflammatory activation of endothelial cells and the subsequent release of these mediators has been associated with the development of atherosclerosis, hypertension and heart failure (Olszanecki et al., 2006).

While H₂O₂ stimulation is shown to directly upregulate NO production in HUVEC, some studies suggest a protective effect of this NO over-production (Liu et al., 2009). NO is an important regulator of cardiovascular homeostasis that is synthesised by endothelial NO synthase (eNOS) in the vasculature. H₂O₂ has been shown to induce eNOS gene expression and increase NO production in endothelial cells; this may represent an initial acute compensatory response to increased stress intended to protect the cells rather than produce cell injury (Cai et al., 2003). However, as endogenous antioxidant defenses become overwhelmed by ROS, oxidative stress and inflammation ensues, leading to cellular damage. Pro-inflammatory cytokines, such as TNF-α, have been shown to upregulate inducible NO synthase (iNOS) activity and NO production in HUVEC (Xia et al., 2006). This flux of NO along with the presence of excessive ROS, may favor the reaction of NO with superoxide anion producing peroxynitrite, resulting in increased oxidant stress and a net decrease in NO production (Xia et al., 2006). Interestingly, this downregulation of NO production has also been implicated in the pathogenesis of cardiovascular diseases, as decreased NO is associated with impaired endothelium-dependent vasodilation (Liu et al., 2009).

NF-κB activation is also associated with the production of inflammatory cytokines. Inflammatory cytokines play a role in endothelial cell injury by inducing the expression of cell adhesion molecules (Lee et al., 2011b). Oxidative stress induction with H₂O₂ increased the production of pro-inflammatory cytokines, including IL-2, IL-6, IL-8, IL-17, IFN-γ, and TNF-α in HUVEC. The addition of astaxanthin to cell cultures had mixed effects on inflammatory cytokine production. Pre-incubation with astaxanthin significantly decreased IL-8 and IFN-γ, but increased IL-17 production. IL-8 has been implicated in the pathogenesis of a number of inflammatory diseases, as it is a chemo-attractant and activator for neutrophils (Nyhlen et al., 2004). Additionally, IFN-γ is shown to modulate IL-8 production in HUVEC (Nyhlen et al., 2004). In the present study, astaxanthin appeared to have a protective effect against inflammation by downregulating IL-8 and IFN-γ production.

IL-2, IL-6, IL-8, IFN-γ, and TNF-α are generally classified as Th1-modulating cytokines due to their ability to induce immune responses, while IL-4 and IL-10 are generally classified as inhibitory or Th2-modulatory cytokines due to their ability to inhibit pro-inflammatory cytokines. Th1-modulating cytokines are known to block the proliferation of Th2 cells, leading to an inhibition of Th2 effector function and a decrease in the production of anti-inflammatory cytokines. However, stimulation with H₂O₂ in the present study also led to an increase in the production of the anti-inflammatory cytokines IL-4 and IL-10; pretreatment with astaxanthin didn’t alter this production. IL-4 and IL-10 have been shown to inhibit TNF-α and IL-6 production in isolated mononuclear cells,
while IL-4 stimulated IL-6 production in HUVEC (Guzdek et al., 2000). IL-4 and IL-10 also increased IL-8 production in lipopolysaccharide-stimulated HUVEC (De Beaux et al., 1995). Tissue specific differences in intracellular signaling pathways and complex interactions play critical roles in cytokine production.

In conclusion, astaxanthin accumulates in HUVEC and incubation with this carotenoid protects against inflammation and oxidative stress induced by H$_2$O$_2$. The action of astaxanthin in ROS clearance helps restore cellular antioxidant function and inhibits ligands required for downstream transcriptional activation and inflammatory gene expression. In general, the downregulation of NF-$\kappa$B and AP-1 resulted in the decreased production of inflammatory biomarkers involved in endothelial dysfunction and CVD.

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