



Siphonaxanthin, a carotenoid from green algae, suppresses advanced glycation end product-induced inflammatory responses

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Abstract

Advanced glycation end products (AGEs) induce inflammation and contribute to the pathogenesis of atherosclerosis. Although many studies have demonstrated the protective effects of carotenoids against atherosclerosis, the effects of carotenoids on AGE-induced inflammation have not been characterized. As such, we aimed to identify carotenoids that provided protection against AGE-elicited inflammation. AGE-stimulated RAW264 macrophages were first evaluated for NO generation. Among 17 carotenoids tested, only siphonaxanthin significantly suppressed it. Next, mRNA expression levels were measured in RAW264 macrophages and human umbilical vascular endothelial cells following siphonaxanthin and AGE treatment. Siphonaxanthin significantly suppressed AGE-induced mRNA expression of interleukin-6 and cellular adhesion molecules, which are known to be important for the pathogenesis of atherosclerosis. Siphonaxanthin also significantly suppressed endoplasmic reticulum (ER) stress marker genes. A reporter gene assay revealed that siphonaxanthin, as well as an ER stress inhibitor, significantly inhibited AGE-induced nuclear factor- κ B (NF- κ B) activation. Altogether, mitigation of ER stress and subsequent NF- κ B activation is one of the molecular mechanisms by which siphonaxanthin suppressed AGE-elicited inflammation. Siphonaxanthin is a carotenoid commonly found in standard diets and is considered relatively safe for human consumption, and hence, dietary intake of siphonaxanthin or siphonaxanthin-containing green algae could be beneficial in lowering the risk of developing atherosclerosis.

Keywords Siphonaxanthin · Carotenoid · Advanced glycation end product · Anti-inflammation · Endoplasmic reticulum stress

Introduction

Non-enzymatic glycation between reducing sugars and amino groups of proteins, nucleic acids, and lipids was first described by the French biochemist Louis Camille Maillard in 1912. This reaction, often called the Maillard reaction, is known to occur during food processing and storage, and leads to the formation of advanced glycation end products (AGEs). AGEs are also produced in vivo and accumulate in the vascular wall in normal subjects and to a greater extent

in patients with diabetes. Both exogenously administered and endogenously generated AGEs induce inflammation through binding with receptors for AGEs (RAGE). Upon binding with AGEs, RAGE initiates its own signaling pathway leading to the activation of nuclear factor- κ B (NF- κ B) and subsequent proinflammatory gene expression [1]. AGE-RAGE-mediated inflammation is involved in the pathogenesis of various diseases, including atherosclerosis. Previous studies have shown that blockade of the AGE-RAGE axis using soluble RAGE inhibited development and progression of atherosclerotic lesions in a mouse diabetes model [2, 3]. Based on these results, development of new low molecular weight compounds which suppress AGE-induced inflammation has received increased attention.

Carotenoids are natural lipophilic pigments responsible for yellow to red coloration. Since they are widely distributed in nature, various carotenoids are found in normal diets. The biological activity of carotenoids has been studied extensively, and various human studies have indicated

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that dietary carotenoids exert health benefits. The Los Angeles Atherosclerosis Study indicated an inverse association between plasma carotenoids (lutein, zeaxanthin, β -cryptoxanthin, and α -carotene) and early atherosclerosis [4]. The Rotterdam Study also showed a modest inverse association between serum lycopene and atherosclerosis [5]. In addition to these epidemiological studies, numerous human intervention studies, animal studies, and cell culture studies have demonstrated the relationship between carotenoids and risk of atherosclerosis [6]. Many cell culture models have shown the inhibitory effect of carotenoids on atherosclerosis-related inflammation. However, the effects of carotenoids on AGE-induced inflammatory responses have not been characterized. In this study, we aimed to identify carotenoids that can inhibit the AGE-elicited inflammatory response.

Materials and methods

Reagents

Glycolaldehyde-AGE modified bovine serum albumin (AGE-BSA) was purchased from Bio Vision (Milpitas, CA). The lot number of AGE-BSA was 3E05L22210, and the degree of lysine modification was 33.9%, according to a fluorescamine-based assay [7]. Carotenoids were prepared as previously reported [8, 9]. All other reagent-grade chemicals, media, and solvents were commercially available.

Cell culture

RAW264 cells (RIKEN Cell Bank, Ibaraki, Japan) were grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 100 units/mL penicillin, and 100 μ g/mL streptomycin. THP1-Dual cells (InvivoGen, San Diego, CA) and human umbilical vein endothelial cells (HUVECs, Kurabo Industries, Osaka, Japan) were cultured according to the manufacturer's instructions. All cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂.

Pretreatment with carotenoids

To disperse carotenoids into the culture medium, a dimethyl sulfoxide/tetrahydrofuran (1:2, v/v) mixture (for RAW264 and HUVECs) or dimethyl sulfoxide (for THP1-Dual) was used as a vehicle (the final concentrations were 0.3% and 0.1%, respectively). The cells were cultured in the carotenoid-containing medium for 24 h before AGE stimulation.

Determination of NO generation

AGE-BSA-induced NO generation was determined by measuring nitrite levels in the medium using a Griess reaction assay as described previously [10]. Briefly, RAW264 cells were pretreated with each carotenoid for 24 h, followed by replacement with 750 μ g/mL AGE-BSA in fresh medium. Following incubation, nitrite levels in the supernatant were determined using a Griess reaction assay.

Evaluation of cell viability

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated with 1 mg/mL MTT in culture medium for 30 min. Then, the supernatants were discarded, and 120 μ L of 2-propanol was added to each well to extract the MTT-derived formazan. After an hour of incubation with shaking, the visible absorption at 570 nm was measured.

Reporter gene assay

We used THP1-Dual cells, which is a commercially available transfected THP-1 human monocyte cell line expressing a secreted embryonic alkaline phosphatase (SEAP) upon NF- κ B activation. THP1-Dual cells plated at a density of 2.5×10^5 cells/mL were stimulated with AGE-BSA (100 μ g/mL) for 24 h. Aliquots (20 μ L) of the supernatant were mixed with 100 μ L of QUANTI-Blue (a substrate for SEAP, InvivoGen) solution. After a 45-min incubation period at 37 °C, the visible absorption at 640 nm was measured.

Total RNA extraction and cDNA synthesis

RAW264 cells in 24-well plates or HUVECs in 24-well collagen type I-coated plates were stimulated with AGE-BSA (750 μ g/mL) for 6 h. Total RNA was isolated, and cDNA was synthesized as described previously [8].

Real-time quantitative PCR analysis

Real-time quantitative PCR analysis was performed using a DNA Engine Opticon system (Bio-Rad Laboratories) as described previously [8]. Primer sequences are shown in Supplementary Table S1.

Statistical analysis

All data are represented as mean \pm SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test or Tukey–Kramer test.

Results

Screening of carotenoids for inhibitory effect on AGE-induced NO generation

RAW264, a murine macrophage cell line, is known to produce NO in response to AGEs. We evaluated the effects of carotenoid treatment on AGE-induced NO production. Among 17 carotenoids, only siphonaxanthin significantly inhibited AGE-induced NO generation in RAW264 macrophages (Fig. 1a). Using the MTT assay, we confirmed

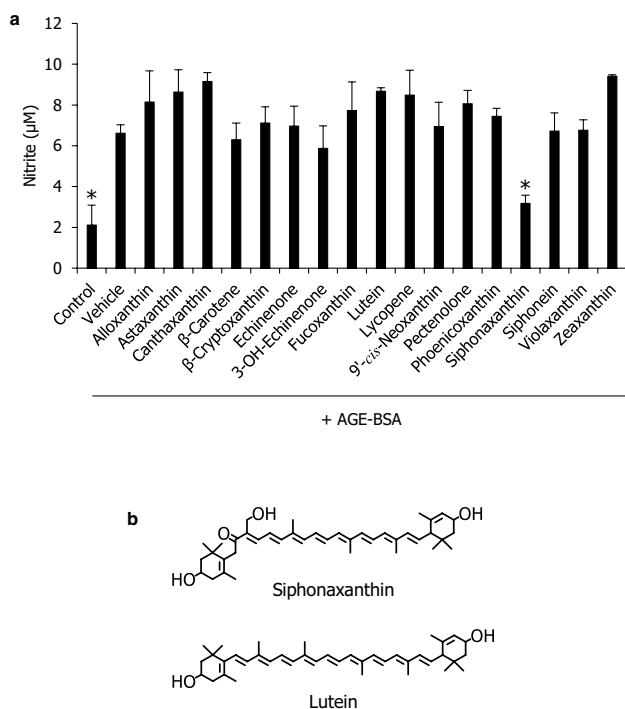


Fig. 1 Result of screening of carotenoids for inhibitory effects on AGE-induced NO generation in RAW264 macrophages. **a** The cells were incubated with 5 μ M of each carotenoid for 24 h, followed by AGE-BSA stimulation. Control and vehicle groups were incubated with the vehicle-containing medium for 24 h, followed by no stimulation or AGE-BSA stimulation, respectively. After 24 h, the supernatants were collected, and nitrite levels were determined using the Griess method. Values are expressed as mean \pm SD ($n=3$). Asterisks indicate values that were significantly lower than those in the vehicle-treated group ($p<0.05$, Dunnett's test). **b** Chemical structure of siphonaxanthin and lutein

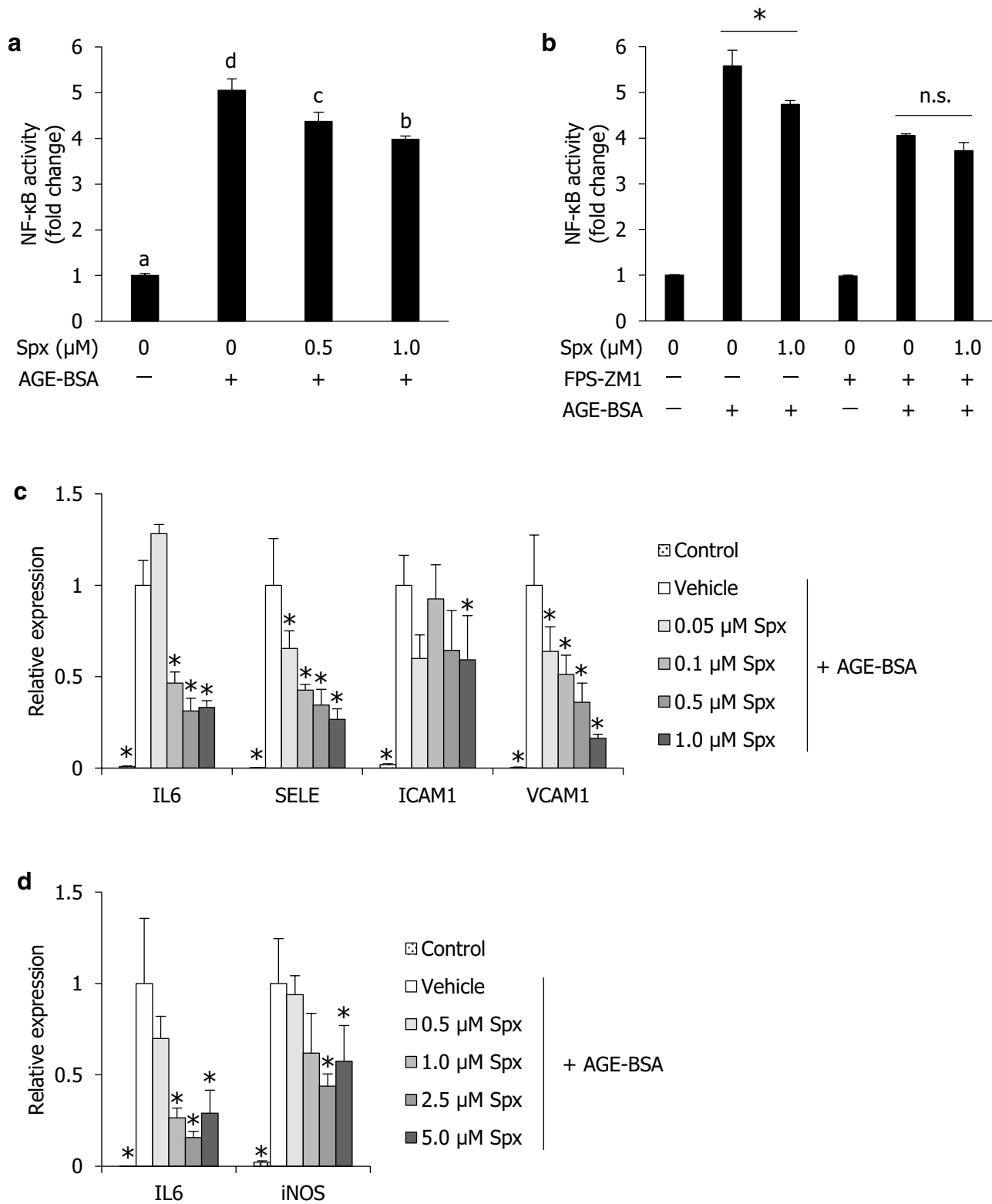
that cell viability was not affected by pretreatment with siphonaxanthin.

Inhibitory effect of siphonaxanthin on AGE-RAGE axis-mediated NF- κ B activation and proinflammatory gene expression

To examine the effects of siphonaxanthin on AGE-RAGE axis-mediated NF- κ B activation, we used THP1-Dual cells, which express SEAP under the control of NF- κ B. Siphonaxanthin significantly inhibited AGE-induced NF- κ B activation in a dose-dependent manner (Fig. 2a). As shown in Fig. 2b, in the presence of FPS-ZM1 (Merck, Darmstadt, Germany), an antagonist of RAGE, siphonaxanthin did not show any significant effect, indicating that siphonaxanthin suppressed AGE-RAGE axis-mediated NF- κ B activation. We also measured NF- κ B-controlled and atherosclerosis-related proinflammatory gene expression in AGE-exposed HUVECs and RAW264. As shown in Fig. 2c, siphonaxanthin significantly inhibited AGE-BSA-induced mRNA expression of interleukin-6 (IL6) and cellular adhesion molecules such as selectin E (SELE), intercellular adhesion molecule 1 (ICAM1), and vascular cell adhesion molecule 1 (VCAM1). In the case of RAW264 murine macrophages, pretreatment with siphonaxanthin significantly suppressed AGE-BSA-induced mRNA expression of IL6 and inducible NO synthase (iNOS) (Fig. 2d).

Mitigation of endoplasmic reticulum (ER) stress and NF- κ B activation

To elucidate the relationship between AGE-induced ER stress and NF- κ B activation, THP1-Dual cells were pretreated with tauroursodeoxycholic acid (TUDCA), a well-known inhibitor of ER stress, and then stimulated with AGE-BSA. Pretreatment with TUDCA significantly inhibited AGE-BSA-induced NF- κ B activation in a dose-dependent manner, indicating that mitigation of AGE-induced ER stress resulted in inhibition of NF- κ B activation (Fig. 3a). THP1-Dual cells were also pretreated with siphonaxanthin in the presence or absence of TUDCA, followed by AGE-BSA-stimulation. TUDCA did not affect the inhibitory effect of siphonaxanthin on AGE-induced NF- κ B activation. The percent of inhibition of siphonaxanthin was $20.1 \pm 4.3\%$ in the absence of TUDCA, $22.1 \pm 4.0\%$ in the presence of 250 μ M TUDCA, and $19.1 \pm 2.9\%$ in the presence of 500 μ M TUDCA (Fig. 3b). We then evaluated the effects of siphonaxanthin on AGE-induced ER stress responses. Messenger RNA for X-box binding protein 1 (XBPI) is alternatively spliced during ER stress, and the spliced mRNA can be used as a marker of ER stress. In HUVECs and RAW264 cells, AGE-BSA treatment increased the levels of spliced XBPI



mRNA. This increase was significantly reversed by pretreatment with siphonaxanthin (Fig. 3c, d). These results indicated that siphonaxanthin mitigated AGE-induced ER stress. Moreover, pretreatment with siphonaxanthin

reduced the increased mRNA expression of HSPA5 and DDIT3, which are known to be increased in response to ER stress (Fig. 3c, d).

Fig. 2 Effect of siphonaxanthin on AGE-RAGE axis-mediated NF- κ B activation and proinflammatory gene expression. **a, b** THP1-Dual cells, which express SEAP under the control of NF- κ B, were pretreated with siphonaxanthin at the indicated concentrations for 24 h, then treated with AGE-BSA in the absence or presence of 25 μ M FPS-ZM1, an antagonist of RAGE. After treatment, activity of SEAP in the supernatant was measured per the manufacturer's instructions. Values are expressed as mean \pm SD ($n=4$). The different characters and the asterisk represent significant difference among treatments, and n.s. represents a difference that was not significant ($p<0.05$, Tukey–Kramer test). HUVECs (**c**) and RAW264 (**d**) were treated with siphonaxanthin at the indicated concentrations for 24 h, followed by stimulation with AGE-BSA for 6 h. The cells in the control and vehicle group were incubated with the vehicle-containing medium for 24 h, followed by no stimulation or AGE-BSA-stimulation, respectively. The mRNA expression of each gene was determined by real-time RT-PCR analysis. Values are expressed as mean \pm SD ($n=4$). Asterisks indicate values significantly different from those in the vehicle group ($p<0.05$, Tukey–Kramer test)

Increase in mRNA expression of several antioxidant genes after the siphonaxanthin treatment

We also evaluated the effects of siphonaxanthin on antioxidant gene expression in HUVEC and RAW264. Twenty-four hours incubation with 1.0 μ M siphonaxanthin significantly upregulated mRNA expression of heme oxygenase 1 (HMOX1) in HUVEC (Fig. 4a). In the case of RAW264, incubation with 5.0 μ M siphonaxanthin for 24 h significantly increased mRNA expression of glutamate-cysteine ligase modifier subunit (GCLM) and thioredoxin reductase 1 (TXNRD1) (Fig. 4b).

Discussion

Siphonaxanthin is a keto-carotenoid distributed in green algae such as *Caulerpa lentillifera* and *Codium fragile*. *C. lentillifera* is known as “sea grape” or “green caviar,” and is a popular seafood in Japan. *C. fragile* was consumed as a part of the staple diet in ancient Japan [11]. Therefore, siphonaxanthin is a carotenoid commonly found in standard diets and is considered relatively safe for human consumption. In the green algae, siphonaxanthin is synthesized de novo with lutein as an intermediate. Therefore, the chemical structures of siphonaxanthin and lutein are similar. Kostic et al. showed that human serum lutein concentrations reached 0.7 μ M after a single oral administration of lutein (0.5 μ mol/kg body weight) [12]. Landrum et al. reported that daily ingestion of 30 mg of lutein for 140 days resulted in constitutively higher serum levels of lutein, and on some days the concentration exceeded 3.0 μ M [13]. As such, we determined that the necessary concentration of siphonaxanthin needed to induce effects in cell culture experiments would be low, as indicated by human serum concentrations of lutein.

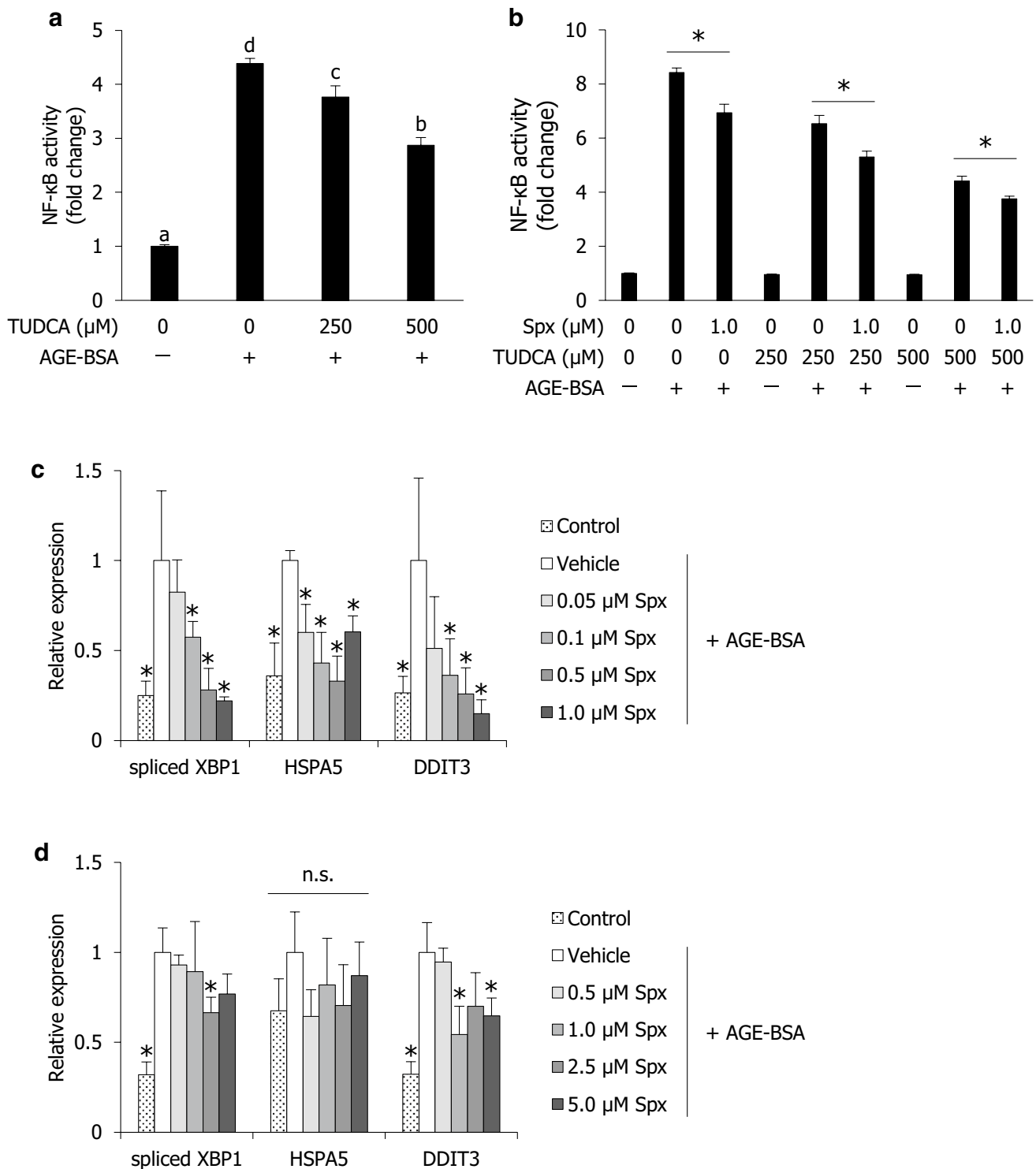
According to our screening results, only siphonaxanthin clearly suppressed AGE-induced NO production in RAW264 macrophages. Compared with lutein, siphonaxanthin has two additional substitutions, a C8-keto group and a C19-hydroxy group. Among the carotenoids we tested, fucoxanthin and siphonaxanthin also have a C8-keto group, whereas no carotenoid but siphonaxanthin has a C19-free hydroxy group. Taken together, a C19-free hydroxy group could be an important chemical structure for the inhibitory effect of carotenoids on AGE-induced inflammatory responses.

Endothelial activation and subsequent adhesion of monocytes is an important series of events in initiation of atherosclerosis. Vascular endothelial cells express cellular adhesion molecules such as SELE, ICAM1, and VCAM1 in response to inflammatory stimuli including AGEs [14]. Inhibiting or eliminating cellular adhesion molecules has been shown to decrease atherosclerotic lesions in animal models [15]. As such, siphonaxanthin might inhibit development of AGE-induced atherosclerotic lesions by inhibiting AGE-induced expression of cellular adhesion molecules.

Chung et al. reported an inverse association between plasma lutein concentrations and IL6 levels in patients with coronary artery disease [16]. They also showed that lutein treatment decreased lipopolysaccharide-stimulated mRNA and protein expression of IL6 in peripheral blood mononuclear cells of patients with stable angina. Based on these reports, we chose IL6 as a marker to evaluate the effects of siphonaxanthin on mRNA expression of proinflammatory cytokines. Pretreatment with siphonaxanthin significantly suppressed mRNA expression of IL6 in both HUVECs and RAW264 macrophages. We also showed that pretreatment with siphonaxanthin significantly suppressed AGE-induced activation of NF- κ B, which is a critical transcription factor that regulates expression of proinflammatory cytokines such as IL6. Siphonaxanthin inhibited AGE-induced expression of proinflammatory cytokines through inhibition of NF- κ B activation.

NO is one of the most important vasodilators in the body. At low physiological levels, NO also acts as an antioxidant. However, local production of large amounts of NO can result in the production of reactive oxidative metabolites such as peroxynitrite. iNOS is an inducible NO synthase, and AGE treatment has been shown to induce iNOS expression via activation of NF- κ B [17]. Furthermore, iNOS expression was increased in macrophages and T-lymphocytes in advanced atherosclerotic plaques. A selective iNOS inhibitor was reported to delay the progression of atherosclerosis [18]. Therefore, siphonaxanthin could delay the progression of atherosclerosis through inhibition of AGE-induced NF- κ B activation, subsequent iNOS expression, and NO generation in macrophages.

Recently, the relationship between ER stress and NF- κ B activation has been studied extensively. In this study, we



showed that the ER stress inhibitor, TUDCA, significantly suppressed AGE-BSA-induced activation of NF-κB. In addition, we found that TUDCA did not affect the inhibitory effect of siphonaxanthin on AGE-induced NF-κB activation. Pretreatment with siphonaxanthin also significantly decreased AGE-induced NF-κB activation and ER stress marker expression. Based on these results, mitigation of

ER stress would be one of the molecular mechanisms by which siphonaxanthin inhibits AGE-induced inflammatory responses.

Wu et al. reported that the elimination of reactive oxygen species (ROS) mitigated AGE-induced ER stress [19]. It is also reported that nuclear factor-erythroid 2-related factor-2 (Nrf2) activation and subsequent increase in

Fig. 3 AGE-induced NF- κ B activation and ER stress responses. **a** THP1-Dual cells, which express SEAP under the control of NF- κ B, were incubated with TUDCA at the indicated concentrations for 24 h, followed by AGE-BSA stimulation. After stimulation, activity of SEAP in the supernatant was measured according to the manufacturer's instructions. Values are expressed as mean \pm SD ($n=4$). The different characters represent significant difference among treatments ($p<0.05$, Tukey–Kramer test). **b** THP1-Dual cells were treated with 1.0 μ M siphonaxanthin in the presence of the indicated concentrations of TUDCA. After the 24-h treatment, the cells received AGE-BSA stimulation, and then, activity of SEAP in the supernatant was measured as described above. Values are expressed as mean \pm SD ($n=4$). HUVECs (**c**) and RAW264 (**d**) were treated with siphonaxanthin at the indicated concentrations for 24 h, followed by stimulation with AGE-BSA for 6 h. The cells in the control and vehicle groups were incubated with the vehicle-containing medium for 24 h, followed by no stimulation or AGE-BSA-stimulation, respectively. The mRNA expression of each gene was determined by real-time RT-PCR analysis. Values are expressed as mean \pm SD ($n=4$). Asterisks indicate values significantly different from those in the vehicle group, and n.s. represents differences which were not significant ($p<0.05$, Tukey–Kramer test)

antioxidant gene expression suppressed AGE-induced inflammatory responses [20]. Moreover, several carotenoids were reported to induce antioxidant gene expression via activation of Nrf2 [21–23]. As such, we evaluated

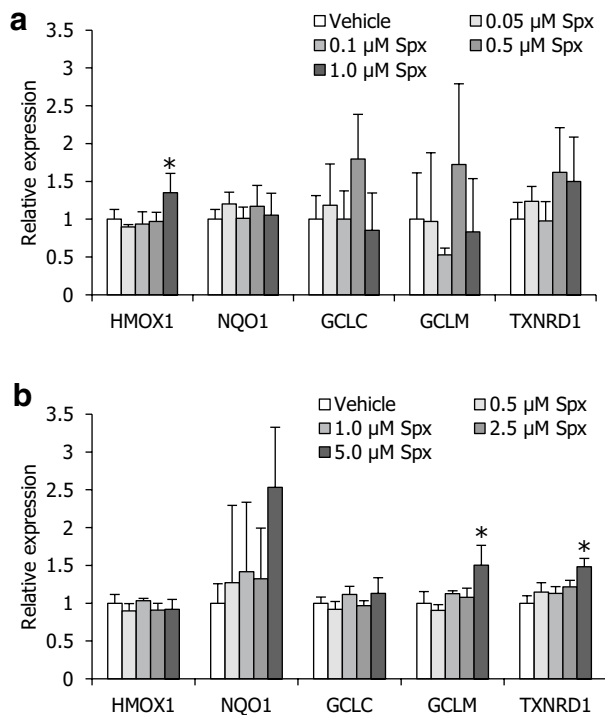


Fig. 4 Effect of siphonaxanthin on antioxidant gene expression. HUVECs (**a**) and RAW264 (**b**) were treated with siphonaxanthin at the indicated concentrations for 24 h. The mRNA expression of each gene was determined by real-time RT-PCR analysis. Values are expressed as mean \pm SD ($n=4$). Asterisks indicate values significantly different from those in the vehicle group ($p<0.05$, Tukey–Kramer test)

antioxidant gene expression in siphonaxanthin-treated cells to elucidate molecular mechanisms underlying mitigation of AGE-induced ER stress by siphonaxanthin. In both HUVEC and RAW264, siphonaxanthin slightly but significantly increased some antioxidant gene expression at the highest doses. However, mitigative effects of siphonaxanthin on AGE-induced ER stress were observed also at lower doses, and hence, other molecular mechanisms might exist. Further studies are required to elucidate molecular mechanisms by which siphonaxanthin mitigates AGE-induced ER stress.

Since various molecules, such as cytokines and oxysterols, are involved in pathogenesis of atherosclerosis, numerous cell culture models have been used to elucidate the protective effects of carotenoids. In this study, we focused on AGE-induced inflammatory responses. According to our results, siphonaxanthin suppressed AGE-induced inflammation, which is a key contributor to pathogenesis of atherosclerosis. Although we only evaluated a small subsection of the inflammatory response, dietary intake of siphonaxanthin or siphonaxanthin-containing algae could be beneficial in lowering the risk of developing atherosclerosis. Future animal and human studies are needed to further evaluate the protective effects of siphonaxanthin against atherosclerosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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