Astaxanthin Enhances ATP-Binding Cassette Transporter A1/G1 Expressions and Cholesterol Efflux from Macrophages

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Summary ATP-binding cassette transporters (ABC) A1 and G1 are key molecules in cholesterol efflux from macrophages, which is an initial step of reverse cholesterol transport (RCT), a major anti-atherogenic property of high-density lipoprotein (HDL). Astaxanthin is one of the naturally occurring carotenoids responsible for the pink-red pigmentation in a variety of living organisms. Although astaxanthin is known to be a strong antioxidant, it remains unclear through what mechanism of action it affects cholesterol homeostasis in macrophages. We therefore investigated the effects of astaxanthin on cholesterol efflux and ABCA1/G1 expressions in macrophages. Astaxanthin enhanced both apolipoprotein (apo) A-I- and HDL-mediated cholesterol efflux from RAW264.7 cells. In supporting these enhanced cholesterol efflux mechanisms, astaxanthin promoted ABCA1/G1 expression in various macrophages. In contrast, peroxisome proliferator-activated receptor γ (PPARγ) liver X receptor (LXR) α and LXRβ levels remained unchanged by astaxanthin. An experiment using actinomycin D demonstrated that astaxanthin transcriptionally induced ABCA1/G1 expression, and oxysterol depletion caused by overexpression of cholesterol sulfotransferase further revealed that these inductions in ABCA1/G1 were independent of LXR-mediated pathways. Finally, we performed luciferase assays using human ABCA1/G1 promoter-reporter constructs to reveal that astaxanthin activated both promoters irrespective of the presence or absence of LXR-responsive elements, indicating LXR-independence of these activations. In conclusion, astaxanthin increased ABCA1/G1 expression, thereby enhancing apoA-I/HDL-mediated cholesterol efflux from the macrophages in an LXR-independent manner. In addition to the anti-oxidative properties, the potential cardioprotective properties of astaxanthin might therefore be associated with an enhanced anti-atherogenic function of HDL.

Key Words astaxanthin, HDL, cholesterol efflux, ABCA1, ABCG1

High density-lipoprotein (HDL) removes cholesterol pathologically accumulated in atherosclerotic lesion macrophages and transports it back to the liver for subsequent conversion to bile in a process called reverse cholesterol transport (RCT) (1). ATP-binding cassette transporters A1 and G1 (ABCA1, ABCG1) play essential roles in cholesterol efflux from macrophages and HDL formation by acting in a sequential manner: ABCA1 generates nascent HDL particles from lipid-poor apolipoprotein A-I (apoA-I) (2), followed by further cholesterol efflux via ABCG1 to form mature HDL particles (3). Deletion of both ABCA1 and ABCG1 in macrophages reportedly accelerated atherosclerotic lesion development as compared to deletion of either (4), indicating that they have a synergetic role in anti-atherogenesis.

ABCA1/G1 in macrophages are transcriptionally regulated by ligand-dependent nuclear receptors: a peroxisome proliferator-activated receptor (PPAR) γ-liver receptor X (LXR) pathway (5, 6). Induction of ABCA1 by PPARγ agonists is completely cancelled in the absence of LXRa/β expression in mice (6), indicating that the effects of PPARγ are dependent on LXRs. Recently, we reported that ABCG1 expression was transcriptionally regulated via both LXR-dependent and -independent pathways (7).

Thus, ABCA1/G1 have drawn much attention as promising therapeutic targets for atherosclerotic diseases. Besides expanding drug discovery, there has been accumulating evidence demonstrating that several nutrients exert beneficial effects on cholesterol efflux from macrophages by increasing ABCA1/G1 expressions. Anthocyanins, a large group of naturally phe-
nolic compounds abundant in plants, enhanced ABCA1 expression by stimulating PPARγ/LXR pathways, thus resulting in increased cholesterol efflux from macrophages. We recently observed that coffee’s phenolic acids, caffeic/ferulic acids, induced ABCG1 and scavenger receptor class B, type I (SR-BI) expression, which is also involved in HDL-mediated cholesterol efflux, leading to increased HDL-mediated cholesterol efflux from macrophages (8). Additionally, in line with the in vitro finding, ferulic acid was found to promote overall RCT in vivo in mice.

Astaxanthin is a red carotenoid pigment found in some seafood, such as salmon, crab, and shrimp. Astaxanthin belongs to the xanthophyll class of carotenoids and is closely related to β-carotene, lutein, and zeaxanthin, sharing the general metabolic and physiological functions attributed to carotenoids. We have previously reported favorable effects of astaxanthin, attenuation of oxidative stress (9) and inhibition of LDL oxidizability by its antioxidative activity (10). However, it remains unclear whether astaxanthin affects cholesterol efflux and ABCA1/G1 expression in macrophages.

Here we demonstrated that astaxanthin increased ABCA1/G1 expression, resulting in enhanced cholesterol efflux from macrophages. Further analyses revealed that astaxanthin transcriptionally promoted ABCA1/G1 expressions in an LXR-independent manner.

MATERIALS AND METHODS

Materials. Astaxanthin was purchased from Alexis (San Diego, CA, USA), and 8-bromo cyclic adenosine monophosphate (8-Br-cAMP), human apoA-I, actinomycin D (ActD) and 22-hydroxycholesterol (22HC) from Sigma (St. Louis, MO, USA), and phorbol 12-myristate 13-acetate (PMA) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HDL was isolated by sequential ultracentrifugation and was prepared according to the methods previously reported (11–13).

Cell cultures. RAW264.7 (RAW) cells (Riken Cell Bank, Tsukuba, Japan) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). THP-1 cells (Riken Cell Bank) were maintained in RPMI 1640 (Sigma) containing 10% FBS. The differentiation of THP-1 monocytes into macrophages was induced in the presence of 320 nmol/L of PMA for 72 h. Mouse peritoneal macrophages were harvested from the peritoneal cavity of mice as described previously (8, 14). This animal study was approved by the Ethics Committee of National Defense Medical College. Briefly, for in vitro experiments, C57BL/6 mice (CLEA Japan, Inc., Tokyo, Japan) were injected intraperitoneally with 10% thioglycollate (Difco, Detroit, MI, USA). Three days later, the mice were euthanized and macrophages collected by lavage of the peritoneum with ice-cold phosphate buffered saline (PBS). For in vivo experiments, resident macrophages were collected without injection of thioglycol-
late. Cells were seeded on 12-well plates at a density of 2x10^6 cells/well in DMEM plus 10% FBS.

Determination of cholesterol efflux. Cholesterol efflux experiments were performed as previously described (11–13). In brief, RAW cells or THP-1 macrophages were radiolabeled with 3H-cholesterol (1.0 μCi/mL) in media containing 0.2% bovine serum albumin (BSA) for 24 h, and in the case of RAW cells, in the presence of 0.3 mmol/L 8-cAMP. The cells were washed twice with PBS and replenished with DMEM containing 0.2% BSA plus the indicated doses of astaxanthin or the vehicle. The cells were again washed with PBS and incubated in the presence or absence of apoA-I (10 μg/mL) or HDL (50 μg/mL), and incubated for 24 h. The percentage of cholesterol efflux was calculated by dividing the media-derived radioactivity by the sum of the radioactivity in the media and the cells. The within-assay coefficient of variation of the cholesterol efflux assay was 5.5%.

Western blot analyses. Cells were harvested and protein extracts were prepared as previously described (11–13). They were then subjected to Western blot analyses (10% SDS-PAGE; 25 μg protein per lane) using rat anti-ABCA1 antiserum (kindly donated by Dr. S. Yokoyama of Nagoya City University) (15), and rabbit anti ABCG1 (Novus Biologicals, Littleton, CO, USA), LXRα (PP-PPZ0412-00, Perseus Proteomics, Tokyo, Japan), LXRβ (PP-K8917-00, Perseus Proteomics), PPARγ(s), Santa Cruz, Santa Cruz, CA, USA) and β-actin (sc-47778, Santa Cruz)-specific antibodies. The proteins were visualized and quantified using a chemiluminescence method (ECL Plus Western Blotting Detection System; GE Healthcare, Bucks, UK) and the NIH image analysis software program.

Real-time quantitative RT-PCR. At the indicated hours after treatment with PPARγ agonists or other compounds, total RNA was extracted from the cells, and first-strand cDNA was synthesized from the total RNA (500 ng) with a Reverse Transcription Reagent (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed with a Perkin-Elmer 7900 PCR machine, TaqMan PCR master mix and FAM-labeled TaqMan primers and probes (Assays-on-Demand, Applied Biosystems) for mouse ABCA1, ABCG1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression data were normalized for GAPDH levels.

Cloning and generation of recombinant adenoviruses encoding for mouse sulfotransferase family cytosolic 2B member 1. A recombinant adenovirus expressing mouse cholesterol sulfotransferase family cytosolic 2B member 1 (Ad-mSult2b1) was produced using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. Briefly, to generate an entry clone of the Gateway Adenoviral Expression System (Invitrogen), cloning of the open reading frame into a pENTR/D-TOPO vector (Invitrogen) was carried out using first-strand complementary DNA derived from mouse skin as a template and the specific primers as follows: forward: 5′-CACCATGGACGCGCCCGCAAGC-3′; reverse: 5′-TATATTGTGAGGATCCTGGTGGG-3′.
An expression clone for adenoviral vector was then generated by performing an LR recombination reaction between the entry clone and a pAd/CMV/V5-DEST (Invitrogen) according to the manufacturer’s protocol. The recombinant adenoviral plasmid was purified, and then transfected into 293A cells. After a sufficient cytopathic effect had been observed in the 293A cells, the adenovirus was purified using the Adeno-X Virus Purification Kit (Clontech, Palo Alto, CA, USA). Adenoviral vector expressing luciferase (Ad-Luc) was kindly donated by Dr. S. Santamarina-Fojo of the National Institutes of Health (NIH), and used as a control. The adenovirus titer in plaque-forming units was determined by a plaque formation assay following infection of HEK293 cells. The multiplicity of infection (MOI) was defined as the ratio of the total number of plaque-forming units to the total number of cells that were infected.

Construction of luciferase reporter plasmids. The human ABCA1 promoter region spanning −940 to +110 bp (hABCA1−940+110) was PCR-amplified using ABCA1-specific primers with HindIII sites (forward: 5′-CCCAAGCTTAAGTTGGAGGTCTGGAGTGG-3′; reverse: 5′-CCCAAGCTTACCGGCTCTGTTGGTGCGCGG-3′) and a plasmid containing the nucleotides −2245 to +110 of the human ABCA1 promoter region derived from the human BAC clone as a template (kindly provided by Dr. S. Santamarina-Fojo of the NIH). The PCR-amplified product was ligated into a pGL3 Basic vector (Promega, Madison, WI, USA) and confirmed by DNA sequencing. To obtain a reporter construct with mutations in the direct repeat 4 (DR4) element (designated as DR4mut) within the LXR-responsive element (LXRE) of the ABCA1 promoter, we performed site-directed mutagenesis using a Quick Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the respective primers: forward: 5′-CGAGCGCAGAGGTTACTATCTG-CAGAAGCCTGTGCTCTCCC-3′; reverse: 5′-GGGAGAGC-ACAGGCTTCTGCAGATAGTAACCTCTGCGCTCG-3′. Luciferase reporter plasmids that contain the human ABCG1 promoter B spanning −1180 to +144 bp (upstream of exon 5, hABCG1B−1180+144) were generated as previously reported (16). The intronic DNA sequence LXRE-A, which was reported to be LXRE with DR4 (Fig. 4C) (17), was PCR amplified from human genomic DNA with primers: forward: 5′-GCTGTAAGCCACAGTGCAT-3′; reverse: 5′-TGTCTGCGGCCCCAT-TCTAT-3′. Luciferase reporter plasmids that contain the human ABCG1 promoter B spanning −1180 to +144 bp (upstream of exon 5, hABCG1B−1180+144) were generated as previously reported (16). The intronic DNA sequence LXRE-A, which was reported to be LXRE with DR4 (Fig. 4C) (17), was PCR amplified from human genomic DNA with primers: forward: 5′-GCTGTAAGCCACAGTGCAT-3′; reverse: 5′-TGTCTGCGGCCCCAT-TCTAT-3′. The amplified products were cloned into the SaII site downstream of the firefly luciferase gene in the hABCG1B−1180+144 plasmid (hABCG1B-LXRE-A). We prepared the LXRE-B region (47 bp), which is located in intron 7 (Fig. 4C), with SaII ends and with or without mutations in DR4 as synthetic complementary oligodeoxynucleotides, and then cloned these regions (hABCG1B-LXRE-B/DR4mut). The oligodeoxynucleo-
otide sequence for LXRE-B was 5′-CCGCCCGCCG-GGTTACTACCGGTCAACGCTCGCTAGTAACCTCC-3′.

DNA transfection and luciferase assays. RAW cells, which had been cultured in 24-well plates, were transfected with 500 ng of luciferase reporter plasmids and 12.5 ng of phRL-TK (Promega) per well using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer’s instructions. Ten hours after transfection, the media were replaced with DMEM containing astaxanthin, 22HC, or the vehicle and incubated for an additional 24 h. Luciferase assays were performed as previously described (11, 13).

Statistical analysis. Statistical analyses were performed using the Stat View Version 5.0 software package (SAS Institute Inc., NC, USA). One-factor factorial ANOVA followed by the Bonferroni/Dunn post-hoc test was used, with a value of \( p < 0.05 \) considered to be significant. All results were expressed as the mean±SD.

RESULTS

Astaxanthin promotes apoA-I- and HDL-mediated cholesterol efflux and ABCA1 and ABCG1 expression in macrophages

As shown in Fig. 1A and B, we first investigated whether astaxanthin affected cholesterol efflux from mouse RAW cells. The results revealed that astaxanthin dose-dependently promoted both apoA-I- and HDL-mediated cholesterol efflux from RAW cells (up to 116 and 25%, respectively). In the absence of apoA-I/HDL, astaxanthin did not alter cholesterol efflux to the media (0.2% BSA containing DMEM, data not shown). To examine underlying molecular mechanisms, we determined ABCA1, ABCG1 and SR-BI expressions. Figure 1C and D show that treatment with astaxanthin dose-dependently brought about up to 2.0 and 3.2 fold increases in protein expressions of ABCA1 and ABCG1, respectively, but not SR-BI. We also observed time-dependent effects of astaxanthin on protein levels of ABCA1 and ABCG1 in RAW cells (Fig. 1E).

Astaxanthin enhances ABCA1 and ABCG1 expression in various macrophages, and does not affect PPARγ/LXR expressions

We next performed Western blots for PPARγ/LXR, which transcriptionally regulate ABCA1/G1 expression as described above. Figure 2A demonstrates that there were no significant changes in LXRα, LXRβ or PPARγ protein expressions in RAW cells. We further confirmed that astaxanthin promoted ABCA1/G1, but not SR-BI expressions in human-derived THP-1 cells (Fig. 2B) or mouse peritoneal macrophages (Fig. 2C) at a concentration of 50 and 25 μmol/L or greater, respectively, indicating that this stimulatory effect of astaxanthin on ABCA1/G1 levels was universal among humans and mice, and primary cells and cell lines. In mouse peritoneal macrophages, astaxanthin did not affect LXRα, LXRβ, or PPARγ protein expressions.

Astaxanthin transcriptionally induces ABCA1 and ABCG1 expression via LXR-independent pathways

We next determined mRNA levels of ABCA1/G1, revealing that, in parallel with increased protein levels, astaxanthin dose-dependently increased ABCA1/G1 mRNA levels (Fig. 3A). To further explore the mechanisms for increased ABCA1/G1 levels, we investigated whether astaxanthin transcriptionally induced these genes using ActD, an inhibitor of transcription. As shown in Fig. 3B, ActD abolished the inducible effects of astaxanthin on ABCA1/G1 expression in RAW cells, suggesting that astaxanthin did transactivate these genes. For the experiments using mouse macrophages, 8-Br-cAMP was added in order to induce ABCA1 expression. Therefore, we also performed the same experiment in the presence of 8-Br-cAMP. Figure 3C shows that astaxanthin again exerted the stimulatory effects on ABCA1 levels, which were dramatically
enhanced to 46-fold by 8-Br-cAMP compared to the vehicle. In contrast, ActD completely cancelled the induction of ABCA1 expression by 8-Br-cAMP, and also the effect of astaxanthin. These data indicate that astaxanthin transcriptionally induces ABCA1/G1 expressions in macrophages.

Next, to further assess the role of LXRs in astaxanthin-mediated effects on ABCA1/G1, we used an adenovirus vector encoding a mouse oxysterol catabolic enzyme, cholesterol sulfotransferase (Ad-mSult2b1), to inactivate LXR signaling by depleting oxysterols, which are LXR ligands (18). We confirmed that Sult2b1 overexpression completely cancelled the increased expression of ABCA1 and ABCG1 induced by oxysterol 22HC as expected (Fig. 3D). In contrast, the stimulatory effects of astaxanthin on ABCA1/G1 levels were similarly observed either in the presence or absence of Ad-mSult2b1, indicating that astaxanthin induces ABCA1/G1 expression in macrophages in an LXR-independent fashion.

**Astaxanthin transcriptionally induces ABCA1 and ABCG1 expression via LXR-independent pathways**

Finally, we investigated the effects of astaxanthin on human ABCA1/G1 promoter activities. Since DR4 in LXRE is a pivotal sequence for LXR binding among human ABCA1 promoter, we introduced mutations in DR4 (Fig. 4A). For the wild type DR4, 100 μmol/L of astaxanthin activated ABCA1 promoter by 1.9 fold, which was comparable to 22HC, an LXR agonist. In the presence of mutation in DR4, the induction of ABCA1 promoter activity by 22HC was cancelled as previously reported (7); in contrast, astaxanthin maintained its stimulatory effect (Fig. 4B).

Figure 4C shows the genomic organization and reported transcripts (19, 20) of human ABCG1. We recently investigated the relative contributions of promoters A and B (located upstream of exons 1 and 5, respectively) in macrophages and found that the activity of promoter B was substantially greater than that of promoter A (7). Therefore, to investigate the mechanisms for transcriptional regulation of human ABCG1 by astaxanthin, we focused on promoter B.

Figure 4E clearly demonstrates that insertion of LXRE-A or -B downstream of the luciferase gene (Fig. 4D) conferred 22HC-responsiveness in human ABCG1 promoter B activity. In contrast, it revealed that astaxanthin resulted in dose-dependent enhancement of promoter B activity either in the presence or absence of LXREs.

Taken together, these observations suggest that astaxanthin enhanced ABCA1/G1 expression by activating the promoters in an LXR-independent manner.

**DISCUSSION**

Plasma concentrations of HDL-cholesterol have strong inverse correlations with risk of atherosclerotic cardiovascular disease (21). Although the mechanism by which HDL may exert a direct protective effect against development of atherosclerosis is not yet well understood, HDL has been postulated to facilitate the efflux of cholesterol from peripheral tissues and transport it back to the liver in a process called RCT (1).
Recently several key molecules have been identified to play pivotal roles in RCT (2, 3, 22). ABCA1 facilitates cholesterol efflux from cells to lipid-poor apoA-I, but not HDL (2, 22). While another ABC transporter, ABCG1, and SR-BI are involved in the cholesterol efflux from macrophages mediated by HDL, apoA-I is not involved in this process (3, 22, 23). Here we showed, for the first time, that astaxanthin enhanced cholesterol efflux from macrophages by increasing ABCA1 and ABCG1 expression. Promoter assays further revealed that astaxanthin activated ABCA1 and ABCG1 promoters in an LXR-independent manner.

Astaxanthin is a more potent antioxidant and scavenger of free radicals than carotenoids such as β-carotene. These functions, together with anti-inflammatory properties, have potential as a therapeutic...
agent in atherosclerotic disease. Several clinical studies showed favorable data on safety, bioavailability, oxidative stress, and inflammation (24). Furthermore, several animal studies have also shown favorable effects of astaxanthin or synthetic astaxanthin on pathological conditions underlying cardiovascular diseases, namely lowering of blood pressure (25), anti-diabetic effects (26), and attenuation of myocardial infarction size (27, 28). However, it has yet to be determined whether astaxanthin brings about beneficial effects on surrogate endpoints for clinical cardiovascular outcomes. In this regard, future studies are needed to investigate these issues in the human setting. Concentrations of dietary nutrients should be high in the intestine, where ABCA1 is reportedly highly expressed. Brunham et al. (29) reported that approximately 30% of the steady-state plasma HDL pool is contributed by intestinal ABCA1 in mice. Therefore, it is possible that astaxanthin not only promotes RCT through macrophages, but also raises HDL levels, as reported in a human study (30).

The present study sheds new light into the potential anti-atherogenic properties of astaxanthin in addition to anti-oxidative and anti-inflammatory functions. Whether the increased cholesterol efflux by astaxanthin is attributed to its anti-oxidative property remains inconclusive. For example, probucol, a potent antioxidant, decreases cholesterol efflux due to increased degradation of the ABCA1 protein in macrophages (31, 32). Likewise, a-tocopherol, a fat-soluble antioxidant, disturbs LXR mediated regulation of ABCA1, ABCG1 and cholesterol handling in macrophages (33). Overall, it is conceivable that an antioxidant property does not always translate into the increased cholesterol efflux of HDL.

LXR plays important roles in the transcriptional regulation of genes involved in cellular cholesterol homeostasis. Oxysterols and 9-cis-retinoic acid activate transcription by binding to LXR and retinoid X receptor, respectively, to form heterodimers which, in turn, bind to conserved LXRE containing DR4 in the ABCA1 (34) and ABCG1 (16, 17, 20) promoter regions. We recently reported that a PPAR-y agonist, pioglitazone, induced LXRα expression, leading to ABCA1/G1 transactivation, and thereby promoting cholesterol efflux to both apoA-I and HDL from macrophages (12, 35). In the present study, we found that 1) astaxanthin transcriptionally induced ABCA1/G1 expression in macrophages and that 2) astaxanthin did not affect PPAR-y or LXR levels in RAW cells (Fig. 2B) or mouse peritoneal macrophages (Fig. 2D). Furthermore, based on the experiment where oxysterol depletion was induced by Sult2B1 overexpression, astaxanthin exerted its stimulatory effect on ABCA1/G1 expression even though the LXR-pathway was shut off (Fig. 3D). Finally, the present study clearly demonstrated that LXREs were not involved in induction of ABCA1/G1 transactivation by astaxanthin. Overall, these results favor a concept that astaxanthin activates ABCA1/G1 promoters in an LXR-independent manner. However, LXR has been shown to increase expressions of its target genes in a ligand-independent fashion by forming a heterodimer with RXR (36). Moreover, RXR expression is reportedly increased by astaxanthin in neural stem cells (37). To exclude this possibility, further investigations, such as electrophoretic mobility shift assay or chromatin immunoprecipitation assay, are needed. It remains unclear how astaxanthin transactivates ABCA1/G1; however, previous papers reported that ABCA1 was transcriptionally regulated via LXR-independent pathways, such as USF (38), AP2 (15), sp1 (39), and so on. Our ABCA1 promoter construct also contains these regulatory cis elements; therefore future experiments are needed using ABCA1/G1 promoter constructs with serial deletions to explore astaxanthin-responsive elements.

In conclusion, astaxanthin has an anti-atherogenic property via HDL by increasing ABCA1 and ABCG1 expression and enhancing apoA-I/HDL-mediated cholesterol efflux from the macrophages. This novel function of astaxanthin may be exerted in concert with the anti-oxidative properties to provide a potential cardioprotective benefit in humans.

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