Inhibitory Effect of Carotenoids on Ligand-induced Lipid Raft Translocation of Immunoreceptors

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Abstract: Lipid rafts are microdomains present in the plasma membrane, which are enriched in sphingolipids and cholesterol. Certain kinases and adaptor proteins, which are important for cellular signaling, are also concentrated in lipid rafts. Several immunoreceptors are known to translocate into lipid rafts upon binding with their ligands to efficiently induce the signaling pathways, and hence, receptor translocation could be the new target for pleiotropic suppression of inflammatory responses. In this study, we evaluated the effects of carotenoids on ligand-induced lipid raft translocation of the receptors using B cell receptors (BCRs) as a model. Since all lipid raft-translocated BCRs were clustered at one pole of the cell, called capping, in our experimental condition, we screened the carotenoids for their inhibitory effect on lipid raft translocation of receptors using BCR capping as a parameter. Eleven out of twenty carotenoids significantly inhibited anti-IgM-induced BCR capping without cytotoxicity. Having no polar groups or a keto group at the C-8 position might be an important factor for inhibition. Treatment with lycopene, a non-polar carotenoid, and fucoxanthinol, a C-8-keto carotenoid, also suppressed lipopolysaccharide-induced translocation of Toll-like receptor 4 into lipid rafts, and subsequent nitric oxide production in RAW264 macrophages. These results indicated that some carotenoids, but not all, can modulate inflammatory responses via suppression of ligand-induced lipid raft translocation of immunoreceptors, and also showed that our assay using BCR capping has the potential for screening compounds that inhibit lipid raft translocation of receptors.

Key words: carotenoid, receptor mobilization, lipid raft, B cell receptor, capping formation

1 INTRODUCTION

Carotenoids are one of the most widespread natural pigments and are produced by photosynthetic organisms and fungi. Over 750 carotenoids have been identified thus far, and about one-third of them are of marine origin[1]. The tetraterpenoid structure with a long-conjugated polyene chain confers potent anti-oxidant activity and lipophilic characteristics to carotenoids (Fig. 1). Substituent groups, such as hydroxy, epoxy, or carbonyl groups, and structural elements, such as allenic or acetylenic bonds, account for its great structural diversity. These functional groups, which are often found in marine-derived carotenoids, are considered to be important for their biological activities. For example, a hydroxy group at the C-3 position of the e-end group was found to be essential for the inhibition of phorbol ester-induced Epstein-Barr virus activation in a lymphoma cell line[2]. An allenic bond was reported to be important for suppressing prostate cancer cell proliferation[3] and adipocyte differentiation[4]. An acetylenic bond was shown to be an important structure for suppression of superoxide and nitric oxide (NO) generation from leukocytes[5]. We also previously reported that a hydroxy group at the C-19 position is important for inhibition of adipocyte differentiation[6]. This essential information about chemical structures for biological activities could help us predict the activities of new or rare carotenoids.

The molecular mechanisms underlying the biological activities of carotenoids have also been studied extensively. Especially, the relationship between carotenoids and some nuclear receptors has been well studied. Astaxanthin was reported to act as an agonist of peroxisome proliferator-activated receptor (PPAR)α and an antagonist of PPARγ[7]. Lutein activated retinoic acid receptor (RAR)β in RAW264.7 macrophages[8] and lycopene induced activation of PPARγ and liver X receptor (LXR)α in THP-1-derived macrophages[9]. β-Cryptoxanthin increased CYP27A1
Fig. 1  Chemical structures of carotenoids.
mRNA expression level, which was partially decreased in the presence of pan-antagonist for RARs\[^{10}\]. We also previously reported that siphanoxanthin significantly suppressed LXR activation induced by its synthetic agonist\[^{11}\]. On the other hand, due to their lipophilicity, carotenoids are considered to be oriented in the plasma membrane, and their biological activities in the membrane have also received particular attention. In fact, using model bilayer membranes consisting of phosphatidylcholine, zeaxanthin was reported to span across the bilayer\[^{12}\] and effectively protect phosphatidylcholine from oxidation initiated in both aqueous and lipid phases\[^{13}\]. In the thylakoid membrane of higher-plant chloroplasts, membrane fluidity was affected depending on the composition of carotenoids\[^{14}\]. In the membrane of Acholeplasma laidlawii, carotenoids are reported to act as a rigid insert reinforcing the bilayer\[^{15}\]. Using human dermal fibroblasts, Camera \textit{et al.} found that pretreatment with astaxanthin suppressed ultraviolet (UV) A irradiation-induced decrease in plasma membrane integrity\[^{16}\]. We also reported an effect of carotenoids on the plasma membrane of cultured mammal cells. Astaxanthin, β-carotene, fucoxanthin, and zeaxanthin suppressed antigen-induced translocation of type I high affinity IgE receptor (FceRI) into lipid rafts, and as a result, these four carotenoids totally suppressed antigen-induced mast cell activation\[^{17}\].

Lipid rafts are microdomains in the plasma membrane and contain high concentrations of sphingolipids, cholesterol, and a variety of important proteins involved in signaling pathways\[^{18}\]. Various receptors are known to translocate into lipid rafts upon binding with their own ligands, and hence lipid rafts are considered as platforms for efficient signaling initiation. As the importance of lipid rafts in inflammatory responses has been unveiled, modulation of lipid raft function has become one of the targets to elucidate the molecular mechanisms of anti-inflammatory food ingredients. For example, cyanidin-3-glucoside was reported to suppress CD40L-induced tumor necrosis factor receptor-associated factor (TRAF)-2 recruitment to lipid rafts and the subsequent production of inflammatory cytokines\[^{19}\]. Fu \textit{et al.} showed that glycyrrhizin suppressed lipopolysaccharide (LPS)-induced inflammatory responses by inhibition of Toll-like receptor 4 (TLR4) translocation into lipid rafts\[^{20}\]. As described above, we also previously reported that astaxanthin, β-carotene, fucoxanthin, and zeaxanthin suppressed FceRI translocation into lipid rafts\[^{17}\]; however, the effect of other carotenoids and the chemical structures important for the suppression remained unclear.

B cell antigen receptors (BCRs) are known to translocate into lipid rafts upon binding with antigen or anti-IgM antibody in both cell lines and primary B cells\[^{21}\]–\[^{25}\]. Furthermore, BCRs are known to cluster on lipid rafts at one pole of the cell, called capping\[^{26}\]. Hence, we decided to use BCR as a model, and screened twenty carotenoids for their inhibitory effect on lipid raft translocation of BCRs by immunofluorescence staining and subsequent counting on micrographs, and further tried to evaluate the chemical structures important for modulation of lipid raft function.

2 EXPERIMENTAL PROCEDURES

2.1 Cells, antibodies, and other reagents

The human B-cell lymphoma cell line Ramos (Health Science Research Resources Bank, Osaka, Japan) and mouse macrophage raw macrophage RAW264 (RIKEN Cell Bank, Tsukuba, Japan) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO\(_2\). Biotin-conjugates of affinity-purified F(ab\(^\prime\))\(_2\) fragments of goat anti-human IgM were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Fluorescein isothiocyanate (FITC)-conjugated anti-human CD55 antibody was purchased from Exbio (Vestec, Czech Republic). Anti-mouse TLR4 antibody (H-80) and LPS from Escherichia coli 0111:B4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated anti-rabbit IgG antibody was purchased from Millipore (Billerica, MA). Alexa 594-conjugated cholaera toxin B subunit (CTxB) was obtained from Invitrogen (Carlsbad, CA). Phycoerythrin-conjugated streptavidin was purchased from BioLegend (San Diego, CA). Carotenoids were prepared as described previously\[^{24}\]–\[^{25}\]. The other chemicals, media, and solvents used in the experiments were of commercially available reagent grade.

2.2 Immunofluorescence microscopy

Ramos cells at a density of 1.0×10\(^6\) cells/mL were cultured in 0.1% fatty acid–free bovine serum albumin (BSA) containing RPMI 1640 medium supplemented with each carotenoid or vehicle (tetrahydrofuran, THF) for 4 h. The concentration of each carotenoid was set at 10 μM and the final concentration of THF was below 0.2%. The cells were washed twice with RPMI 1640, followed by stimulation with 10 μg/mL of biotin-conjugates of affinity-purified F(ab\(^\prime\))\(_2\) fragments of goat anti-human IgM for 30 min at 37°C. The non-stimulated control cells were incubated with the same F(ab\(^\prime\))\(_2\) fragments for 30 min on ice. After washing with phosphate buffered saline (PBS) twice, the cells were fixed in 4% paraformaldehyde-PBS for 20 min at 4°C and blocked with 1% BSA containing PBS. Then, the cells were stained with FITC-conjugated anti-human CD55 antibody, followed by staining with phycoerythrin-conjugated streptavidin. Fluorescence images were acquired using a BioRevo BZ-9000 microscope (Keyence, Osaka, Japan). The data were quantified by counting the number of positive cells and presented as a percentage of the total cells.
RAW264 cells ($4.0 \times 10^5$ cells in 8-well chamber slides; ibidi GmbH, Martinsried, Germany) were treated with carotenoids using the same method as described above. After washing twice with RPMI 1640, the cells were stimulated with 50 ng/mL LPS for 5 min. The cells were then washed twice with ice-cold PBS, and incubated with 2 μg/mL Alexa 594-conjugated CTxB in 1% BSA containing PBS for 30 min at 4°C. After washing with PBS twice, the cells were fixed and blocked as mentioned above. Then, the cells were stained with anti-mouse TLR4 antibody and FITC-conjugated anti-rabbit IgG. Fluorescence images were acquired using the BioRevo BZ-9000 microscope.

2.3 Quantification of carotenoids

Ramos cells were treated with fucoxanthin or lutein as described above. After a 4 h incubation, the cells were washed with 10 mM sodium taurocholate in PBS, and then washed twice with PBS. Total lipids were extracted by Folch’s method, and the content of carotenoids was quantified using high performance liquid chromatography with photodiode array (HPLC-PDA), as described previously.

2.4 Determination of NO production by RAW264 macrophages

LPS-induced NO production was determined using Griess method. In brief, RAW264 cells in 96-well plates were stimulated with LPS (50 ng/mL) for 24 h. Aliquots (50 μL) of the supernatants were mixed with an equal volume of 1% sulfanilamide in 5% phosphoric acid and incubated for 10 min at room temperature. Then, 50 μL of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride was added and incubated for another 10 min. The visible absorption ($\lambda = 540$ nm) was measured using a spectrophotometer. An equal volume of the supernatant incubated without sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride was used as the blank. The quantity of nitrite was determined from a standard curve of sodium nitrite.

2.5 Statistical analysis

All data are represented as means ± standard deviation (SD). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test, Student’s t-test, or Dunnett’s test.

**Fig. 2** Effect of fucoxanthin and lutein on anti-IgM-induced lipid raft translocation of BCRs.

A, Ramos cells were incubated with fucoxanthin or lutein (10 μM) for 4 h (the carotenoids were dissolved in THF, with the final concentration of THF in the medium being 0.2%), and then incubated by biotinylated anti-IgM antibody at 37°C for 30 min. Localization of BCR was visualized by phycoerythrin-conjugated streptavidin. CD55, a marker of lipid rafts, was stained using FITC-conjugated antibody. Scale bar, 5 μm. B, the quantified data of lipid raft translocation of BCR by counting the number of the cells like “Veh” in A. Data are represented as the number of positive cells per total cells. Means are calculated from the data of six independent micrographs (more than 150 cells were counted). Values are expressed as the means ± SD. The different characters represent significant difference among treatments ($p<0.05$; Tukey-Kramer test). Cont, resting cells (control); Veh, vehicle; Fxn, fucoxanthin; Lut, lutein. C, Ramos cells were incubated with the carotenoids using the same method as in A and B. Intracellular carotenoids were extracted and quantified by HPLC-PDA. The data are expressed as the means ± SD ($n = 3$). *, significantly different ($p<0.05$, Student’s t-test).
3 RESULTS

3.1 Fucoxanthin inhibited anti-IgM-induced translocation of BCR into lipid rafts

We previously reported that fucoxanthin, but not lutein, inhibits chemical mediator release from antigen-stimulated mast cells\(^{17, 25}\), and moreover showed that the inhibitory mechanism is the inhibition of antigen-induced FcεRI translocation into lipid rafts\(^{17}\). Hence, we hypothesized that fucoxanthin also inhibits anti-IgM-induced BCR translocation into lipid rafts. As shown in Fig. 2A, after incubation with anti-IgM at 37°C for 30 min, BCR and CD55 (a marker of lipid rafts) localized together, indicating that BCR translocated into lipid rafts in response to anti-IgM stimulation. In fucoxanthin-treated cells, BCR and CD55 did not localize together; however, in lutein-treated cells, co-localization was observed (Fig. 2A). These data indicated that fucoxanthin, but not lutein inhibited anti-IgM induced BCR translocation into lipid rafts. We also quantified the BCR translocation into lipid rafts by counting the number of the cells looking like vehicle-treated cells in Fig. 2A. Treatment with fucoxanthin significantly decreased the percentage of cells that showed BCR translocation, while that with lutein did not have any significant effect (Fig. 2B). We also quantified the cellular uptake of the two carotenoids. Lutein was significantly more accumulated than fucoxanthin (Fig. 2C), indicating that their cellular uptake did not correlate with their inhibitory activities. No fucoxanthinol, a de-acetylated form of fucoxanthin, was detected in this experimental condition.

3.2 The effect of carotenoids on anti-IgM-induced BCR capping formation

It is reported that anti-IgM stimulation not only induces BCR translocation into lipid rafts, but also induces clustering of BCR at one pole of the cells, called capping\(^{23}\). As shown in Fig. 2A, in our experimental condition, anti-IgM-stimulation induced BCR capping, and it was easy to distinguish between the BCR capping-positive and -negative cells. Hence, we decided to use capping as a marker of BCR lipid raft translocation and quantified the effect of carotenoids on anti-IgM-induced BCR translocation by counting the number of BCR capping-positive cells. Twelve out of 100 cells were counted as positive for each condition. The data were expressed as a percentage of vehicle-treated cells. Values represent the means ± SD. The asterisks indicate significant difference compared to vehicle (\(p<0.05\), Dunnett’s test).

![Effect of carotenoids on anti-IgM-induced BCR capping](image-url)
of twenty carotenoids significantly inhibited BCR capping, and lycopene showed the most potent inhibitory effect (Fig. 3). Siphonaxanthin also showed a potent inhibitory effect, but also induced morphological changes in the cells, indicating that treatment with siphonaxanthin could lead to cell death. Such morphological changes were not observed in cells treated with other carotenoids. We also analyzed the inhibitory effect of all carotenoids on anti-IgM-induced BCR lipid raft translocation by co-staining for CD55. Carotenoids that significantly inhibited BCR capping also suppressed BCR lipid raft translocation (data not shown).

3.3 Lycopene and fucoxanthinol inhibited LPS-induced lipid raft translocation of TLR4

Since lycopene showed the most potent inhibitory effect on anti-IgM-induced BCR capping, we next investigated its inhibitory effect on the lipid raft translocation of other receptors. Upon binding to LPS, TLR4 is known to translocate into lipid rafts, and LPS-TLR4 signaling leads to inflammatory responses, such as NO production in macrophages. Hence, we evaluated the effect of lycopene on LPS-induced TLR4 translocation into lipid rafts and subsequent NO production. As shown in Fig. 4A, TLR4 and GM1 (a marker of lipid rafts) were co-localized after LPS stimulation, indicating that LPS induced TLR4 translocation into lipid rafts. On the other hand, in lycopene-treated cells, TLR4 and GM1 did not co-localize, indicating that lycopene suppressed LPS-induced TLR4 translocation into lipid rafts. Lycopene also significantly inhibited LPS-induced NO production (Fig. 4B). Not only non-polar carotenoids, but also carotenoids with a keto group at the C-8 position significantly inhibited BCR translocation into lipid rafts (Fig. 3). Hence, we evaluated the effect of fucoxanthinol, a C-8-keto carotenoid, on LPS-induced lipid raft translocation of TLR4. Fucoxanthinol suppressed TLR4 translocation and subsequent NO production (Fig. 4A, B).

4 DISCUSSION

To our knowledge, this is the first screening report on the inhibitory effect of carotenoids against receptor translocation into lipid rafts, one of the most upstream events of the cellular signaling pathway. Lycopene showed the most
potent inhibitory effect on BCR translocation into lipid rafts and also suppressed LPS-induced TLR4 translocation. β-Carotene, another non-polar carotenoid, also showed a potent inhibitory effect on BCR translocation. Hence, lack of polar groups might be an important factor in the inhibition of lipid raft translocation of receptors. Due to their lipophilicity, non-polar carotenoids are considered to be oriented horizontally in lipid bilayers and increase the fluidity of the plasma membrane. Since lipid rafts are rigid microdomains in the plasma membrane, we speculated that non-polar carotenoids might inhibit the function of lipid rafts by increasing the fluidity of the plasma membrane. Halocynthiaxanthin, fucoxanthin, fucoxanthinol, and siphonelin also showed potent inhibitory effects, and fucoxanthinol suppressed LPS-induced lipid raft translocation of TLR4. These results indicated that the presence of a keto group at the C-8 position might be an important feature of polar carotenoids for the inhibition of receptor translocation. In contrast to non-polar carotenoids, polar carotenoids are considered to span the membrane with interaction between the polar substituents on the end groups and the polar head groups of phospholipids. Hence, we speculated that C-8-keto carotenoids also spanned the plasma membrane with their keto group present in the inner part of the bilayers. Electrostatic repulsion between the keto groups might hinder the tight packing of carotenoid molecules in the plasma membrane and affect the fluidity of the membrane. This might be one of the reasons why C-8-keto carotenoids affect the function of lipid rafts.

According to our results, we also assumed that the substitution of hydroxy group at C-3 position could be another important factor for the inhibition of BCR capping. β-Carotene effectively inhibited the anti-IgM-induced BCR capping, followed by β-carotene (mono-hydroxylated β-carotene) and zeaxanthin (di-hydroxylated β-carotene) in this order. Phoenicoxanthin tended to inhibit the capping formation more potently than astaxanthin, which has an additional hydroxy group at C-3 position. However, echinenone and canthaxanthin did not show the significant inhibition though their mono-hydroxylated form, 3-hydroxyechinenone and phenoxyechinenone, respectively, could suppress the capping significantly. In addition, the substitution of keto group at C-4 position could also affect the activity. β-Carotene more potently inhibited the BCR capping than echinenone, which has an additional keto group at C-4 position, and a comparison of diotaxanthin and pectenolone showed the same result (p<0.05, Tukey-Kramer test). 3-Hydroxyechinenone and phenoxyechinenone have one and two additional keto group at C-4 and C-4', respectively, compared to β-carotoxanthin, and their inhibitory activities decreased as follows: β-carotoxanthin > 3-hydroxyechinenone > phenoxyechinenone. Taken together, lack of polar substituents at end groups of carotenoids appears to be an important factor for the inhibition of anti-IgM-induced BCR capping.

Due to their lipophilicity, it is not easy to dissolve or disperse carotenoids in culture medium, and hence, choosing the vehicle is very important for evaluation of biological activities of carotenoids. In the present study, we used THF as a vehicle because it was reported to be a good solvent for β-carotene, and Bertram et al. reported that to dissolve carotenoids in THF was effective in delivering to cultured cells. Furthermore, we showed that both C-8-keto carotenoids and non-polar carotenoids significantly suppressed the BCR capping. Among twenty carotenoids we used, C-8-keto carotenoids have high polarities, and of course, non-polar carotenoids have low polarities. Therefore, we concluded that our results did not reflect the solubility of each carotenoid in the medium.

In this study, we also quantified the cellular uptake of carotenoids. Lutein was significantly more accumulated than fucoxanthin, but lutein did not affect the lipid raft translocation of BCRs, while fucoxanthin significantly suppressed it. Hence, the inhibitory activity did not correlate with the cellular uptake of carotenoids. Bertram et al. reported that the inhibitory effect of carotenoids on chemically induced neoplastic transformation did not correlate with their uptake, and showed that carotenoids with a potential for conversion to retinoids exhibited high inhibitory activities even if their cellular uptake was low. Wiegandt et al. reported that retinoic acid modulated the metabolism of glycosphingolipids, which are one of the components of lipid rafts. Therefore, it is possible that carotenoid metabolites, such as retinoids could be active forms of carotenoids. However, in this study, the cellular incubation time with carotenoids was only four hours, and deacetylation of fucoxanthin was not observed in Ramos cells under this experimental condition. Hence, we speculated that no metabolic conversion of carotenoids occurred, and the active form could be an intact form of each carotenoid. Bayer et al. reported that both oleanolic acid and its isomer, ursolic acid, similarly accumulated in lipid rafts, but only oleanolic acid could modulate the function of lipid rafts. They discussed that this functional difference between the isomers could be due to their difference in capacity for tight packing with sphingolipids, one of the major components of lipid rafts. Hence, evaluation of lipid raft orientation and capacity of each carotenoid for tight packing is required to further assess the biological activities of carotenoids.

This study also presented a new easy method to obtain anti-inflammatory compounds that can suppress cellular signaling by modulating immunoreceptor lipid raft translocation. Various immunoreceptors, including T cell antigen receptor (TCR), BCR, FceRI, and all TLRs (except for TLR3) are known to translocate into lipid rafts where they initiate their own signaling pathways. Since receptor lipid raft translocation is one of the most upstream signaling events, compounds that can suppress receptor translo-
cation have the potential to inhibit the signaling initiated from these receptors, thereby exerting a strong inhibitory effect. Furthermore, such compounds have been shown to suppress various inflammatory signaling pathways initiated by receptor translocation. Nakahira et al. showed that heme oxygenase-1-derived carbon monoxide inhibited TLR2, 4, 5, and 9 signaling through inhibition of translocation of these TLRs into lipid rafts\(^{39}\). Docosahexaenoic acid (DHA) was reported to suppress LPS-induced translocation of TLR4 into lipid rafts\(^{39}\). Wang et al. reported that n-3 polyunsaturated fatty acids, including DHA, reduced FcεRI association with lipid rafts and mast cell activation\(^{39}\). In the present study, we showed that lycopene and fucoxanthin suppressed the translocation of BCR and TLR4 into lipid rafts. Similarly, compounds that inhibit receptor translocation could inhibit not only one receptor-initiated signaling pathway, but also other signaling pathways involved in receptor translocation; hence, these compounds showed pleiotropic anti-inflammatory effects. In other words, TCR-initiated T cell inflammatory response, BCR-initiated B cell inflammatory response, FcεRI-initiated mast cell inflammatory response, and TLRs (except for TLR3) -initiated macrophage inflammatory responses could all be inhibited by one compound via inhibition of receptor translocation. Therefore, our screening assay suggests a new anti-inflammatory compound that can exert strong pleiotropic effects by inhibiting receptor lipid raft translocation.

Our method presented in this study was based on visual counting on the micrographs. Since anti-IgM antibodies induced BCR clustering at one pole of the cell, called capping, and it is easy to distinguish between the capping-positive and -negative cells, we used BCR capping formation as a model for ligand-induced receptor translocation. Visual counting makes it possible to simultaneously detect the cytotoxicity of compounds by checking for any morphological changes in the cells. Hence, our method is useful for seeking anti-inflammatory compounds. If we could adapt this method to automated cell image analysis, an increasing number of anti-inflammatory compounds with an inhibitory effect on receptor translocation could be identified via high-throughput screenings.

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**Conflict of Interest:**

The authors have no conflict of interest to declare concerning this study.

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