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RESEARCH ARTICLE

β-Carotene and β-cryptoxanthin but not lutein evoke redox and immune changes in RAW264 murine macrophages

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The mechanism of immunological benefits induced by carotenoids has not been fully elucidated. Here, we investigated some of the immunity-related properties of β-carotene and two other carotenoids, β-cryptoxanthin, and lutein, on the murine macrophages cell line RAW264. β-Carotene added to the culture medium accumulated in the cells in a time- and dose-dependent manner. The accumulation was positively correlated with cellular lipid peroxidation, demonstrating the pro-oxidative activity of β-carotene, and also with the synthesis of glutathione, an intracellular antioxidant. Conversely, accumulation of β-carotene was negatively correlated with the transcription of immune-active molecules, such as IL-1β, IL-6, and IL-12 p40, in cells stimulated by LPS and INF-γ. The transcription of the pro-inflammatory cytokines IL-1β and IL-6 was more sensitive to the accumulation of β-carotene than was IL-12 p40. The accumulation of β-cryptoxanthin in cells resulted in effects similar to those of β-carotene. However, lutein accumulated minimally and did not significantly affect the cells. These results demonstrate that β-carotene, and β-cryptoxanthin as well, can accumulate in RAW264 cells and induce changes in intracellular redox status, which in turn regulate the immune function of macrophages.

Keywords:
β-Carotene / β-Cryptoxanthin / Lutein / Macrophage / Redox

1 Introduction

Carotenoids are a family of colored tetra-terpenoids, which generally have a symmetric or quasi-symmetric structure and are hydrophobic. Some of them are pro-vitamin A, which is oxidatively cleaved to vitamin A by β-carotene-15,15′-monooxygenase [1]. Fruits and vegetables are rich in carotenoids, which protect the plants from oxidative stress produced during photosynthesis. *In vitro* studies have demonstrated that carotenoids are very effective quenchers of singlet oxygen [2, 3]. Conversely, it is unclear whether carotenoids play a similar role in animals [4]. Epidemiological studies have shown that high intake of fruits and vegetables negatively correlates with the risk of various human illnesses including cancers and cardiovascular diseases [5, 6], but the physiological benefits of carotenoids, apart from their role as vitamin A precursors, has not been established.

Phagocytes such as macrophages and neutrophils generate and secrete reactive oxygen species (ROS), which

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Abbreviations: BHT, butylated hydroxytoluene; **GCL,** glutamate-cysteine ligase; **GSH,** glutathione; **GSSG,** glutathione disulfide; **ROS,** reactive oxygen species; **TBARS,** thiobarbituric acid-reactive substances

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are responsible for their bactericidal activity. Such phagocyte-derived extracellular ROS are autotoxic, causing inhibition of chemotaxis, phagocytosis, and antimicrobial activity [7]. Thus, to maintain the function of phagocytes, high antioxidant activity is needed for protection against severe oxidative stress [8]. The importance of intracellular redox status on cytokine production by macrophages or antigen-presenting cells has been demonstrated [9–11].

The effect of carotenoids on immune function has been the subject of longstanding discussion. The effect is at least partially independent of pro-vitamin A activity because canthaxanthin, a non-pro-vitamin A carotenoid, showed similar immunological effects of β-carotene [12]. Not a few human intervention studies have examined the effect of supplementation with carotenoid(s) as for immune function. For example, Watzl *et al.* reported time-delayed modulation of immune functions in healthy men in response to vegetable juice consumption [13]. Conversely, a review article summarized that there appeared to be only a few consistent findings among human intervention studies: healthy subjects do not show either enhancement of lymphocyte proliferation or IL-2 production following supplementation with β-carotene [14].

Previously, we compared the effects of β-carotene and α-tocopherol, both of which are known as fat-soluble antioxidants, added to the culture media of RAW264 cells, a murine macrophage cell line. The study showed that α-tocopherol, but not β-carotene, protected the cells from lipid peroxidation elicited by free radicals generated from the Fenton reaction. Conversely, β-carotene, but not α-tocopherol, induced considerable changes in cytoplasmic redox status, which resulted in the enhancement of intracellular antioxidative potential detected using 2′7′-di-chlorofluorescin diacetate [15].

In the present study, to explore the redox- and immune-active properties of β-carotene, we analyzed the kinetics and time course of changes in redox related and immunological indices induced by β-carotene in RAW264 cells. We also investigated the effects of two other carotenoids, β-cryptoxanthin and lutein, to further elucidate the structure–activity relationship for antioxidant activity.

2 Materials and methods

2.1 Chemicals

Non-essential amino acids, penicillin-streptomycin, L-glutamine, Moloney murine leukemia virus reverse transcriptase and dithiothreitol were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was purchased from Equitech-Bio (Kerrville, TX, USA). Minimum essential medium, β-carotene, β-apo-8′-carotenal, 1-fluoro-2, 4-dinitrobenzene, and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Perchloric acid was purchased from Nacalai Tesq (Kyoto, Japan). 1,1,3,3-tetra-ethoxypropane was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Iodoacetic acid was purchased from Wako Pure Chemical Industries (Osaka, Japan). INF-γ was purchased from Genzyme (Cambridge, MA, USA). RNAqueous^{TM} kit was purchased from Ambion (Austin, TX, USA). Oligo $(dT)_{15}$ primer and dNTP mixture were purchased from Promega (Madison, WI, USA). SYBR Green was purchased from Takara BIO (Otsu, Japan). β-Cryptoxanthin and lutein were purchased from Extrasynthese (Genay, France). Dehydrated THF with 0.025% butylated hydroxytoluene (BHT), thiobarbituric acid, and the other organic chemical reagents were purchased from Kanto-chemical (Tokyo, Japan).

2.2 Preparation of culture medium

When adding carotenoids to culture medium, choosing a suitable solvent can be problematic. Bertram *et al.* demonstrated that dehydrated THF containing BHT was an optimal solvent for carotenoids when adding them to the test medium of a cultured cell line [16]. THF has also been used as solvent for carotenoids in other studies using cell lines [17–20]. Therefore, in the present study, carotenoids were dissolved in THF stabilized with 0.025% BHT to prepare a stock 10 mM solution; the stock solution was then added to a standard medium consisting of minimum essential medium supplemented with 10% fetal bovine serum, L-glutamine, non-essential amino acids, and 50 000 U/L penicillin-streptomycin. Solvent without carotenoids was also added to the standard medium for the preparation of the control medium. The final concentration of THF was 0.2%. The stock solution was stored in the dark at −70°C with N_2. The medium containing carotenoids was prepared just before its use because carotenoids are not so stable in the medium. The test medium contained 2.5, 5, or 10 μM carotenoids. Although this concentration range is higher than the reported average concentration of human plasma carotenoids [21, 22], high concentrations comparable to our test medium have been reported among subjects who had taken supplemental β-carotene [23–25]. Media containing similar carotenoid concentrations have been used in many prior studies with cultured cell lines [16–20].

2.3 Cell culture

RAW264 cells were provided by Riken cell bank (Tsukuba, Japan). The cells were grown in standard medium in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For experiments, 3.0×10^6 cells were cultured in plastic dishes (60 mm diameter) containing 5 mL of test media supplemented with various concentrations of β-carotene, β-cryptoxanthin or lutein. Harvested 1.0×10^6 cells, or harvested whole cells from dishes in some experiments, were analyzed to measure various parameters as described in Sections 2.4–2.8. In experiments to detect rapid alternation, 1.0×10^6

cells were incubated with test media in centrifuge tubes and centrifuged whole cells were analyzed. Supplementation with carotenoids did not affect cell proliferation during incubation for up to 24 h (data not shown).

2.4 Quantification of carotenoids

After culturing in test media supplemented with various concentrations of carotenoids, cells were washed with PBS containing 10 mM taurocholate and harvested. As an internal standard β-apo-8′-carotenal was added to harvested cells. The hydrophobic fraction of cellular contents containing carotenoid was extracted with 2.5 mL of hexane/isopropanol (3:2 v/v) containing 0.4 mM BHT. After centrifugation at 3000 rpm at 4°C for 5 min, the upper layer was collected and dried under a stream of N_2. Each residue was dissolved in 100 μL of the solvent: a mixture of acetonitrile, methanol, dichloromethane, and water (7:7:2:0.16) [26]. An aliquot of 20 μL of each solution was injected into a HPLC system with a TSK-gel (Tosoh; Tokyo, Japan) Octyl-80Ts column (ϕ4.6 × 250 mm). The carotenoid content was quantified at a wavelength of 450 nm.

2.5 Quantification of thiobarbituric acid-reactive substances

The content of thiobarbituric acid-reactive substances (TBARS) was determined based on the method of Mihara and Uchiyama [27] with minor modifications, and these values were used as an index of lipid peroxidation. After culturing in test media supplemented with various concentrations of carotenoids, cells were harvested, washed with PBS, and combined with 50 μL of fresh PBS. Cell suspensions were then mixed with 1 mL of 1% phosphate solution containing 50 μL of 10 mM BHT. The suspension was then mixed with 1 mL of 0.67% thiobarbituric acid solution and boiled for 30 min. After cooling, 1 mL of butanol was added and the mixture was centrifuged at 3500 rpm for 5 min at 4°C. The upper layer was collected and fluorescence was measured at an excitation wavelength of 515 nm and emission wavelength of 553 nm. TBARS were expressed as the content of malondialdehyde calculated from a standard curve prepared with 1,1,3,3-tetra-ethoxy-propane.

2.6 Quantification of intracellular glutathione and glutathione disulfide

Intracellular glutathione (GSH) and glutathione disulfide (GSSG) levels were determined using a modified version of the method described by Fariss and Reed [28]. After culturing in test media supplemented with various concentrations of carotenoids, harvested cells were transferred to centrifuge tubes and suspended in 100 μL of PBS. After the addition of 5 μL of 70% perchloric acid solution, the mixture was centrifuged at 10 000 rpm for 3 min at 4°C. Supernatant (80 μL) was collected and combined with an equal volume of 80 mM iodoacetic acid and 120 μL of saturated sodium bicarbonate solution. After incubation in the dark at room temperature, 80 μL of 5% 1-fluoro-2,4-dinitrobenzene in ethanol (Sigma-Aldrich) was added to the mixture, which was incubated for another 4 h under the same conditions. An aliquot of 20 μL of the resultant mixtures were applied to a column of Develosil NH_2-5 (4.6 × 250 mm) (Nomura-Chemical; Seto, Japan) in a normal phase HPLC system equilibrated with 64% methanol. The column was washed and conditioned with a linear gradient from 0.64 to 3.2 M ammonium acetate. The contents of GSH and GSSG were quantified based on the absorbance at 365 nm.

2.7 Quantitative RT-PCR

Total RNA was extracted from 1.0×10^6 cells by an RNAqueous™ kit according to the manufacturer's instructions. Extracted RNA was solubilized with 15 μL of water. Reverse transcription was performed at 43°C in a total volume of 20 μL containing 10 μL of RNA solution, 0.5 mM of dNTP mixture, 0.5 μg of oligo $(dT)_{15}$ primer, 10 mM dithiothreitol, and 10 U of Moloney murine leukemia virus reverse transcriptase. The primer sequences for β-actin were 5′-TTCTTGGGTATGGAATCCTGTGG-3′ and 5′-TGTTGGCATAGAGGTCTTTACGG-3′. The sequences for the glutamate-cysteine ligase (GCL) modifier subunit (which facilitates the enzymatic activity of its counterpart, the GCL catalytic subunit) were 5′-ACCGGGAACCTGCTCAACTG-3′ and 5′-GATTTGGGAACTCCATTCATTCAAG-3′. The sequences for IL-1β were 5′-TCACAGCACATCAACAAGAG-3′ and 5′-CCAGCAGGTTATCATCATCCC-3′. The sequences for IL-6 were 5′-AGCCAGAGTCCTTCAGAGAGATAC-3′ and 5′-TCTTGGTCCTTAGCCACTCCTTC-3′. The sequences for IL-12 p40 were 5′-ACCAAATTACTCCCGGACGGTTC-3′ and 5′-AGACAGAGACGCCATTCCACA-3′. These primers were designed and synthesized by Takara BIO to our specifications. PCR was performed for 1 μL of reverse transcripts with a primer pair and SYBR green using an ABI PRISM 7500 system (Applied Biosystems; Foster city, CA, USA). Relative mRNA expression was determined by the threshold cycle of each mRNA normalized by that of β-actin as a house-keeping gene.

2.8 Stimulation of RAW264cells by LPS and INF-γ

RAW264 cells were cultured in test media supplemented with various concentrations of carotenoids for 24 h and the supernatant was then removed. Cells were further incubated with fresh media containing LPS (final concentration,

100 ng/mL) and INF-γ (final concentration, 10 ng/mL). After the 6 h stimulation period, cells were harvested for the measurement of relative expression of cytokine mRNA (IL-1β, IL-6, and IL-12 p40) by quantitative RT-PCR as described above.

2.9 Statistics

Data are expressed as mean ± SD from triplicate samples. Statistical significances were determined by one-way analysis of variance with Bonferroni/Dunn *post hoc* tests for multiple comparisons or Student's *t*-test for two-sample comparison using Statcel 2 (OMS; Tokyo, Japan). $p < 0.05$ were considered statistically significant.

3 Results

Accumulation of β-carotene in RAW264 cells increased proportionally to the concentration in the culture medium and reached plateau at 6 h, as shown in Fig. 1A. No β-carotene was detected when cells were cultured with medium that was not supplemented with β-carotene. The fact that the amount of β-carotene in the cells corresponded to the concentration in the medium suggests that β-carotene in the culture medium was transferred to the cells *via* a concentration gradient between the medium and plasma membrane. Significant accumulation was detected as early as 0.5 h after the addition of β-carotene, as shown in Fig. 1B.

When the cells were cultured in media supplemented with β-carotene, the amount of TBARS produced during lipid peroxidation was significantly enhanced in a concentration-dependent manner of supplemented β-carotene, as shown in Fig. 2A. Such dependency demonstrated that the addition of β-carotene to the culture medium induced lipid peroxidation in the cells. Peroxidation in the presence of supplemental β-carotene was not detected at 0.5 h but was detected at 1.0 h, as shown in Fig. 2B.

GSH is a representative intracellular thiol antioxidant. It can reduce intracellular pro-oxidants, resulting in the formation of GSSG from two GSH molecules. GSH is a γ-glutamyl peptide consisting of glutamine, cysteine, and glycine, and the synthesis of γ-glutamylcysteine by GCL is the rate-limiting step. The amount of GCL is a critical determinant of the intracellular GSH concentration. When cells were cultured in media supplemented with β-carotene, the amount of both GSH and GSSG in RAW264 cells significantly increased in a concentration-dependent manner, as shown in Figs. 3A and B. Such dependency demonstrated that β-carotene added to the culture medium increased intracellular GSH levels. Significant enhancement of GSH was detected earlier than that of GSSG, as shown in Figs. 3C and D, suggesting that the enhancement of GSSG resulted from the increase in GSH and its subsequent oxidation. Weak but steady time-dependent increases in the contents of GSH and GSSG were observed even in the absence of β-carotene, due to an increase in the number of cells during the incubation. Concomitantly with the increase in GSH by the addition of β-carotene, transcription of a subunit of GCL also significantly increased in a concentration-dependent manner in response to β-carotene 24 h after its addition, as shown in Fig. 4. In supplemental experiments, a significant increase in GCL was evident as early as 3 h after the addition of β-carotene (100 ± 9.1% in the absence of β-carotene *versus* 240 ± 9.7% in the presence of 10 μM β-carotene, $p < 0.01$). It was earlier than the increase in GSH concentration. These results suggest that the increase in intracellular GSH levels in response to β-carotene was attributable to the up-regulation of synthesis.

To evaluate the effect of β-carotene on immune function of macrophages, we quantified the transcripts of three cytokines

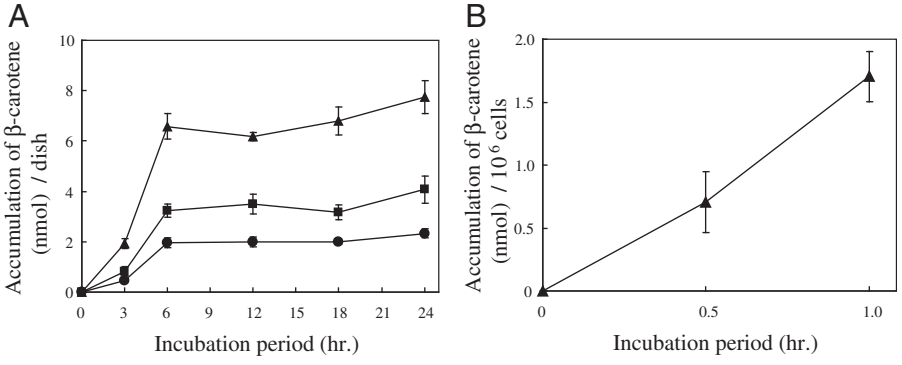

Figure 1. Accumulation of β-carotene in RAW264 cells in response to supplementation with β-carotene to the culture media. RAW264 cells (3.0×10^6) were cultured in plastic dishes (60 mm diameter) with medium containing 2.5 μM (filled circle), 5 μM (filled square), or 10 μM (filled triangle) β-carotene at 37°C for 0, 3, 6, 12, 18, or 24 h (A). Whole cells harvested from the plastic dishes were used to quantify total β-carotene accumulation *per* dish. For detecting rapid accumulation, 1.0×10^6 RAW264 cells were mixed with medium containing 10 μM β-carotene in centrifuge tubes and incubated at 37°C for 0, 0.5, or 1.0 h (B). Whole cells incubated in tubes were centrifuged and β-carotene accumulation in 1.0×10^6 cells was quantified. In both panels, calculated data for whole cells in a dish or a tube are expressed as mean ± SD from triplicate samples.

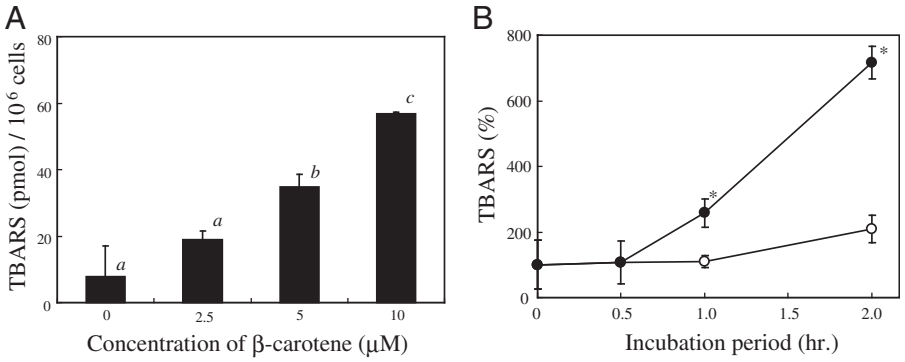

Figure 2. Lipid peroxidation profiles of RAW264 cells in response to supplementation with β-carotene in the culture media. RAW264 cells (3.0×10^6) were cultured in plastic dishes (60 mm diameter) with medium containing 0, 2.5, 5, or 10 μM β-carotene at 37°C for 24 h (A). Harvested 1.0×10^6 cells from the plastic dishes were used to quantify TBARS. Calculated data (TBARS pmol/1.0×10^6 cells) are expressed as mean ± SD from triplicate samples and bars with a different letter are significantly different ($p < 0.05$). For detecting rapid peroxidation, RAW264 cells were cultured with a medium with (filled circle) or without (open circle) 10 μM β-carotene in centrifuge tubes at 37°C for 0, 0.5, or 1.0 h (B). Whole cells centrifuged in tubes were used for the quantification of TBARS. For 2.0 h incubation, RAW264 cells (3.0×10^6) were cultured in plastic dishes (60 mm diameter) at 37°C, and harvested 1.0×10^6 cells from the dishes were used for the quantification of TBARS. Relative TBARS values in comparison with the initial content are expressed as mean percentage ± SD from triplicate samples and filled circles with an asterisk represent significant differences (*$p < 0.05$) compared with the control group (open circles).

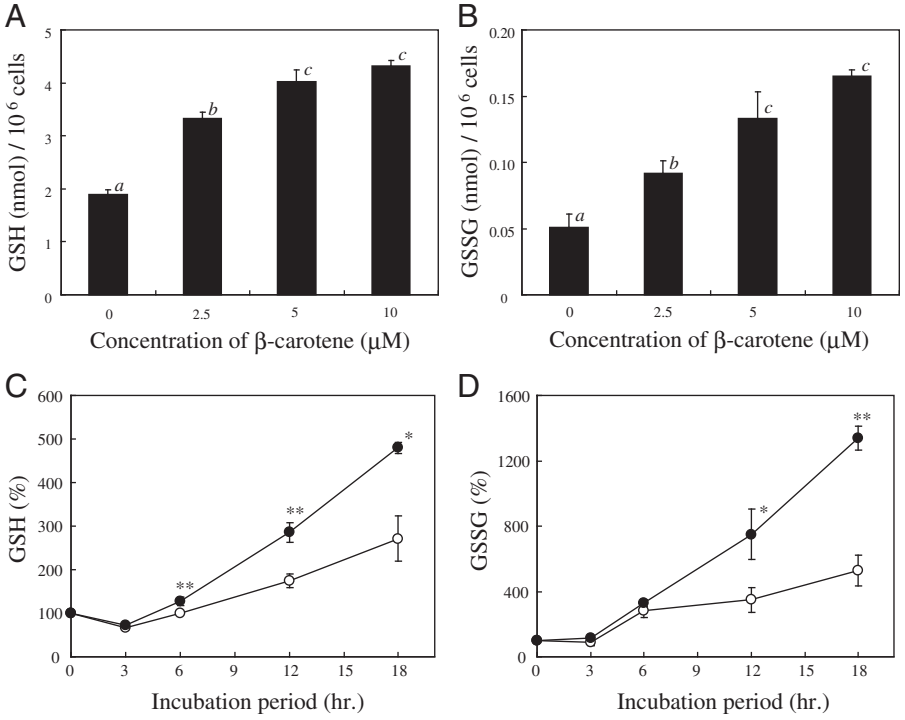

Figure 3. Intracellular GSH and GSSG in response to supplementation with β-carotene in the culture media. RAW264 cells (3.0×10^6) were cultured in plastic dishes (60 mm diameter) with medium containing 0, 2.5, 5, or 10 μM β-carotene at 37°C for 24 h (A and B). Harvested 1.0×10^6 cells from the plastic dishes were used to quantify GSH and GSSG. Calculated data (GSH or GSSG nmol/1.0×10^6 cells) are expressed as mean ± SD from triplicate samples and bars with a different letter are significantly different ($p < 0.05$). RAW264 cells (3.0×10^6) were cultured in plastic dishes (60 mm diameter) in medium with (filled circle) or without (open circle) 10 μM β-carotene at 37°C for 0, 3, 6, 12, or 18 h (C and D). Whole cells harvested from the plastic dishes were used to quantify GSH and GSSG. The relative GSH or GSSG levels in comparison with the initial level are expressed as mean percentage ± SD from triplicate samples, and filled circles with an asterisk represent significant differences (*$p < 0.05$; **$p < 0.01$) in comparison with the control group (open circle).

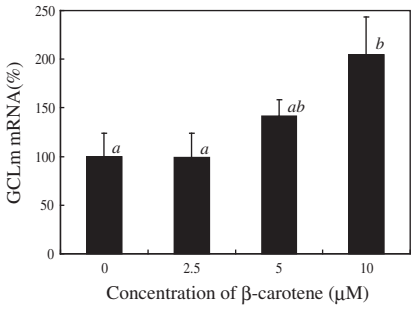

Figure 4. Transcripts of a subunit of GCL in response to supplementation with β-carotene in the culture media. RAW264 cells (3.0×10^6) were cultured in plastic dishes (60 mm diameter) with medium containing 0, 2.5, 5, or 10 μM β-carotene at 37°C for 24 h. Harvested 1.0×10^6 cells from the plastic dishes were used to quantify expressed mRNA of GCL modifier subunit (GCLm). Quantitative RT-PCR was performed, and relative expressions of GCLm mRNA standardized by β-actin mRNA in comparison with the expression in the absence of β-carotene are expressed as mean percentage ± SD from triplicate samples. Bars with different letter are significantly different ($p < 0.05$).

that could be induced by the addition of LPS and INF-γ in RAW264 cells. When the cells were cultured in media supplemented with β-carotene, the transcription of IL-1β, IL-6, and IL-12 p40 was suppressed, as shown in Figs. 5A–C, although the sensitivities differed among the three cytokines. IL-1β transcription was the most sensitive to suppression and IL-12 p40 transcription was the least sensitive to suppression; significant suppression was observed only at the highest concentration of β-carotene. Despite such differences in sensitivity, the transcription of these three cytokines in RAW264 cells showed an overall negative correlation with the accumulation of β-carotene and the content of intracellular GSH.

We next compared the effects of the carotenoids, β-cryptoxanthin, and lutein with those of β-carotene in RAW264 cells. The cellular accumulation of β-cryptoxanthin was comparable to that of β-carotene, as shown in Fig. 6A. β-Cryptoxanthin, like β-carotene, showed redox activity by increasing the levels of TBARS, as shown in Fig. 6B, and by increasing the levels of intracellular GSH, as shown in Fig. 6C. However, its effects appeared to be weaker than those of β-carotene. Lutein that accumulated in RAW264 cells only a little did not show obvious redox activity on either of the indices. β-Cryptoxanthin (10 μM) decreased IL-1β mRNA levels significantly, and decreased IL-6 mRNA levels insignificantly, as shown in Fig. 7. β-Cryptoxanthin did not decrease IL-12 p40 mRNA levels. Lutein did not significantly suppress the transcription of the three cytokines tested.

4 Discussion

Accumulation of intracellular β-carotene; the levels of TBARS, GSH, and GSSG; and the relative mRNA expression of a subunit of GCL, all correlated positively with the β-carotene concentration in culture medium. Accumulation

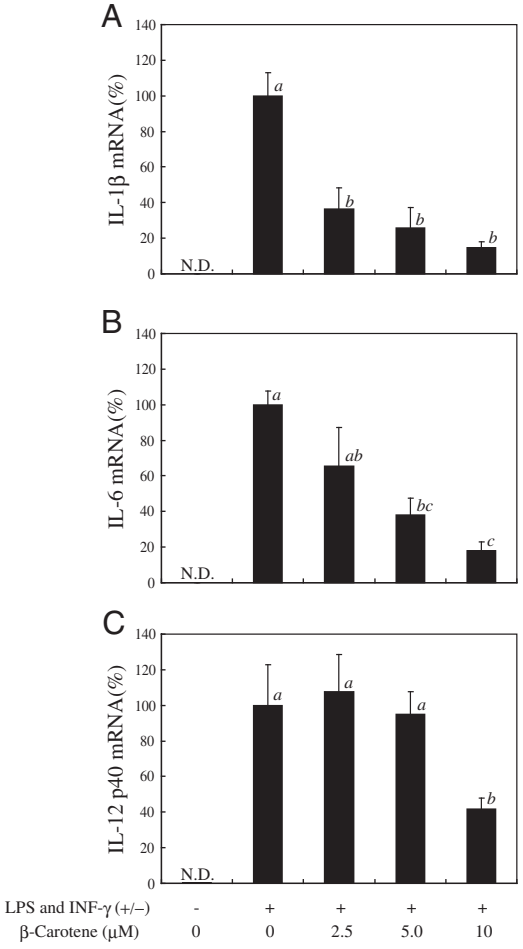

Figure 5. Transcripts of cytokines induced by LPS and INF-γ in response to supplementation with β-carotene in the culture media. RAW264 cells (3.0×10^6) were cultured in plastic dishes (60 mm diameter) with medium containing 0, 2.5, 5, or 10 μM β-carotene at 37°C for 24 h, followed by stimulation with 100 ng/mL LPS and 10 ng/mL INF-γ at 37°C for 6 h. Harvested 1.0×10^6 cells from the plastic dishes were used to quantify mRNA expression for IL-1β (A), IL-6 (B), and IL-12p40 (C). Quantitative RT-PCR was performed, and relative expressions of cytokine mRNA standardized by β-actin mRNA in comparison with the induced expression by LPS and INF-γ in the absence of β-carotene are expressed as mean percentage ± SD from triplicate samples. Bars with a different letter are significantly different ($p < 0.05$). N.D. = not detected.

of β-carotene was detectable at 0.5 h, increased levels of TBARS at 1 h, GCL subunit transcript at 3 h, increased GSH levels at 6 h, and increased GSSG levels at 12 h. The increase in intracellular GSH was not caused by the biological reduction of existing GSSG molecules because the concentration of intracellular GSSG also increased. Furthermore, the increase in the levels of GCL subunit mRNA was detected prior to the increase in GSH. Since the increase in GSSG was detected later than that of GSH, it is likely that the increase in GSH was responsible for the increased GSSG levels, which suggests the consumption of GSH to

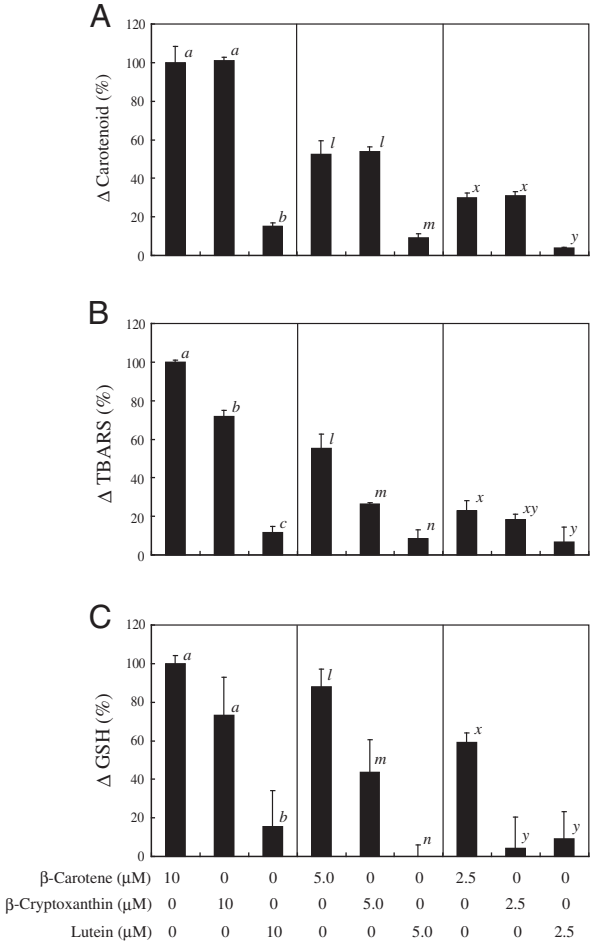

Figure 6. Carotenoid accumulation, lipid peroxidation, and intracellular GSH levels in response to supplementation with various carotenoids in the culture media. RAW264 cells (3.0×10^6) were cultured in plastic dishes (60 mm diameter) with medium containing 2.5, 5, or 10 μM of β-cryptoxanthin, lutein, or β-carotene at 37°C for 24 h. Cells (1.0×10^6) harvested from the plastic dishes were used to quantify carotenoid accumulation (A), TBARS (B), and GSH (C) in the cells. All parameters were measured as described in Section 2. The relative increases in comparison with cells incubated with 10 μM β-carotene are expressed as mean percentage ± SD from triplicate samples. In each panel, bars with a different small letter (*a*, *b*, *c* for cells incubated with 10 μM carotenoid; *l*, *m*, *n* for cells incubated with 5 μM carotenoid; and *x*, *y* for cells incubated with 2.5 μM carotenoid) are significantly different ($p < 0.05$).

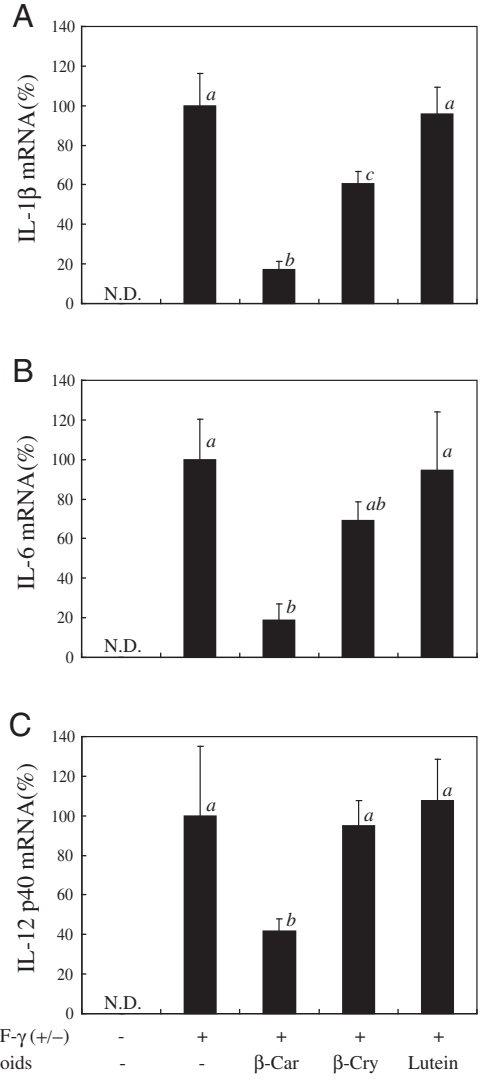

Figure 7. Transcripts of cytokines induced by LPS and INF-γ in response to supplementation with various carotenoids in the culture media. RAW264 cells (3.0×10^6) were cultured in plastic dishes (60 mm diameter) with medium alone or medium containing 10 μM β-carotene (β-Car), β-cryptoxanthin (β-Cry), or lutein at 37°C for 24 h, followed by stimulation with 100 ng/mL LPS and 10 ng/mL INF-γ at 37°C for 6 h. Harvested 1.0×10^6 cells from the plastic dishes were used to quantify the expression of mRNA for IL-1β (A), IL-6 (B), and IL-12p40 (C). Quantitative RT-PCR was performed, and relative expressions of cytokine mRNA standardized by β-actin mRNA in comparison with incubation with LPS and INF-γ in the absence of β-carotene are expressed as mean percentage ± SD from triplicate samples. Bars with a different letter are significantly different ($p < 0.05$). N.D. = not detected.

reduce some intracellular substance. The order of redox-related events taking place following supplementation with β-carotene to the culture medium in this study was assumed to be as follows: (i) β-Carotene is transferred from the culture medium and accumulates in the cell membrane. (ii) β-Carotene acts as a pro-oxidant, directly or indirectly, to induce lipid peroxidation in the cell membrane where β-carotene was incorporated. (iii) Peroxidation of the cell membrane would stimulate intracellular synthesis of GSH and, as a result, intracellular antioxidant capacity rises.

Identifying the molecules responsible for the GCL subunit transcription is of interest; however, we were unable to detect activation of transcription factors such as nrf-2, a representative transcription factor for the antioxidant response element [29], in our system (data not shown). The

mechanism by which GSH synthesis is increased in response to β-carotene awaits clarification in further experiments.

Although the measurement of TBARS as an index of lipid peroxidation is controversial because of low specificity, the method is still widely used. Regardless of the widely accepted status of β-carotene as an antioxidant [2, 3, 30], we detected increased levels of TBARS in cells incubated with β-carotene. However, our TBARS results are consistent with the results of previous studies demonstrating pro-oxidant activity of β-carotene [30–32]. Palozza found that the pro-oxidant effect of carotenoids occurred at relatively high concentrations ranging from 2.5 to 20 μM [33]. Palozza and colleagues also reported the detection of cellular oxidation following accumulation of β-carotene (approximately 1 nmol *per* 10^6 cells) in HL-60 human leukemic cells, after 24 h-incubation with culturing medium containing 20 μM β-carotene [34]. The level of β-carotene detected was similar to the level detected in the present study (Fig. 1) although the effect of β-carotene on GSH levels was different between their study and the present study.

Increased GSH levels accompanied by increased transcription of GCL in response to β-carotene was reported by Ben-Dor *et al.* in a study in MCF-7 (a human mammary cancer cell line) and HepG-2 cells (a human hepatocellular carcinoma cell line); although they did not detect significant decrease in intracellular ROS levels induced by β-carotene [35].

The redox effect of β-carotene appears to vary depending on the type or strain of cultured cells examined. However, we recently showed that GSH synthesis was positively correlated with the accumulation of β-carotene in splenocytes isolated from mice fed diet supplemented with various doses of β-carotene [36].

Expression of mRNA of cytokines such as IL-1β, IL-6, and IL-12 p40 induced by LPS and INF-γ showed a negative correlation with the concentrations of β-carotene in culture media. Since LPS stimulation is transmitted intracellularly *via* ROS [37], intracellular antioxidants can inhibit this signal transduction. For example, addition of *N*-acetyl-L-cysteine, a thiol antioxidant, to culture medium reduces the expression of cytokines induced by LPS [38, 39]. Since the GSH/GSSG ratio, which can be calculated from Fig. 3, was not increased in our experiments (data not shown), it cannot be definitely concluded that the cytoplasmic redox status of RAW264 was reduced by β-carotene. However, increased intracellular GSH, an endogenous intracellular thiol antioxidant, must have played a crucial role in reducing the transcription of cytokines in RAW264 cells. The present study offers compelling evidence that β-carotene affects immune function through the modulation of redox status in macrophages.

In our experiments there was a difference in sensitivity to β-carotene among the cytokines tested: suppression of IL-12 p40 was milder than that of the pro-inflammatory cytokines IL-1β and IL-6. We previously reported that the intake of β-carotene with supplemental α-tocopherol resulted in increased IL-12 p70 secretion from murine splenocytes in response to antigen *ex vivo* in the absence of an increase in IL-12 p40 secretion [40]. The mild suppression of IL-12 p40 expression in response to β-carotene in the present study may explain this inconsistency. β-Carotene may attenuate inflammatory processes triggered by IL-1β and/or IL-6 *in vivo* without significant suppression of antigen-induced adaptive immunity mediated by IL-12.

The carotenoids β-cryptoxanthin and lutein, both of which belong to xanthophyll family of oxygen-containing carotenoids, showed distinctive cellular uptake; β-cryptoxanthin accumulation was similar to that of β-carotene, whereas lutein accumulated very low. β-Cryptoxanthin showed weaker redox effects than β-carotene and, in contrast to β-carotene, it only decreased IL-1β mRNA levels significantly, while lutein had little or no effect on redox or immunological indices. Like β-carotene but in contrast with lutein, β-cryptoxanthin serves as a vitamin A precursor. However, the observed effects of β-carotene and β-cryptoxanthin probably did not depend on vitamin A activity because retinol was not detected in RAW264 cells after incubation with culture medium supplemented with β-carotene, and the mRNA for β-carotene-15,15′-monooxygenase, which catalyzes the production of retinoids from β-carotene or β-cryptoxanthin, was not detected in RAW264 cells (data not shown). Although Rafi and Shafaie reported that lutein could modulate inducible nitric oxide synthase expression in RAW264 cells [41], we did not detect any biological activity of lutein on RAW264 cells within our test parameters. Differences in experimental conditions (*i.e.* they used ethanol as a solvent for carotenoids while we used THF) may explain the conflicting results. The absence of effects of lutein in our experiments may be due to the fact that the cellular accumulation of lutein is markedly lower than the other two carotenoids. The effects of β-carotene on RAW264 cells were detected after it had accumulated in the cells, while the carotenoids that remained in the medium would be unlikely to have affected the cells. β-Cryptoxanthin has an oxygen atom on only one of its ionone rings, while lutein has oxygen atoms on both ionone rings and β-carotene has no oxygen atoms (Fig. 8). Oxygen atoms in the molecular structure may hinder the ability to penetrate the hydrophobic

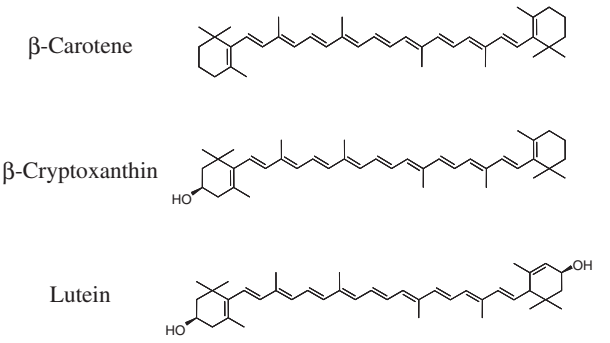

Figure 8. Structural formulas of β-carotene, β-cryptoxanthin, and lutein.

cell membrane and may consequently determine the effect of carotenoids.

Carotenoids present in foods are widely thought to have beneficial effects on health. Here, we demonstrated the modulation of intracellular redox status by β-carotene and β-cryptoxanthin *in vitro*, which in turn regulate the immune function of macrophages. This modulation may play a key role in the health benefits of carotenoids, *e.g.* anti-inflammation resulting in anti-carcinogenesis.

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