Seco-type β-Apocarotenoid Generated by β-Carotene Oxidation Exerts Anti-inflammatory Effects against Activated Macrophages

Naoki Takatani¹, Fumiaki Beppu¹, Yumiko Yamano², Takashi Maoka³, and Masashi Hosokawa¹*¹

¹ Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido 041-8611, JAPAN
² Laboratory of Organic Chemistry for Life Science, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, JAPAN
³ Research Institute for Production and Development, 15 Shimogamo-morimoto-cho, Sakyo-ku, Kyoto 606-0805, JAPAN

Abstract: β-Apocarotenoids are the cleavage products of β-carotene. They are found in plants, carotenoid-containing foods, and animal tissues. However, limited information is available regarding the health benefits of β-apocarotenoids. Here, we prepared seco-type β-apocarotenoids through the chemical oxidation of β-carotene and investigated their anti-inflammatory effects against activated macrophages. Oxidation of β-carotene with potassium permanganate produced seco-β-apo-8'-carotenal, in which one end-group formed an “open” β-ring and the other was cleaved at the C-7',8’ position. In lipopolysaccharide-stimulated murine macrophage-like RAW264.7 cells, seco-β-apo-8'-carotenal inhibited the secretion and mRNA expression of inflammatory mediators such as nitric oxide, interleukin (IL)-6 and IL-1β, and monocyte chemoattractant protein-1. Furthermore, seco-β-apo-8'-carotenal suppressed phosphorylation of c-Jun N-terminal kinase and the inhibitor of nuclear factor (NF)-κB as well as the nuclear accumulation of NF-κB p65. Notably, since seco-β-apo-8'-carotenal exhibited remarkable anti-inflammatory activity compared with β-apo-8'-carotenal, its anti-inflammatory action could depend on the opened β-ring structure. These results suggest that seco-β-apo-8'-carotenal has high potential for the prevention of inflammation-related diseases.

Key words: seco-β-apo-8'-carotenal, apocarotenoid, β-carotene, anti-inflammation, macrophage

1 Introduction

Carotenoids are lipophilic pigments that are widely distributed in microbes, plants, and animals. These natural pigments comprising eight isoprene units (C40), are characterized by a polyene backbone with long conjugated double bonds and various end-group structures. In microbes and plants, carotenoids are ubiquitous and play essential roles in photosynthesis and photoprotection. Dietary carotenoids are incorporated and accumulated in the bodies of animals, including humans, owing their inability to biosynthesize these compounds innately. Among natural carotenoids, β-carotene, which comprises 11 conjugated double bonds and two cyclized end-groups (β-rings), is one of the most abundant carotenoids in nature. Numerous studies have indicated that β-carotene has various nutritional functions such as pro-vitamin A activity, antioxidant and anti-cancer effects, and the prevention of lifestyle-associated diseases¹⁻⁹. Apocarotenoids are defined as carotenoid derivatives in which the carbon skeleton is shortened and formed via enzymatic and non-enzymatic processes¹⁰. Retinoids, which are well-characterized apocarotenoids generated upon central oxidative cleavage of β-carotene, play crucial roles in many physiological processes such as those of the visual³¹, reproductive⁵, and immune systems⁷. Asymmetric β-apocarotenoids, β-apo-13-carotenone, and β-apo-14'-carotenal have been reported to act as antagonists for nuclear receptors such as retinoic acid receptor (RAR), retinoid X receptor (RXR), and peroxisome proliferator-activated receptor (PPAR) family members. Various β-apocarotenoids have been reported to act as antagonists for nuclear receptors such as retinoic acid receptor (RAR), retinoid X receptor (RXR), and peroxisome proliferator-activated receptor (PPAR) family members.

Abbreviations: LPS, lipopolysaccharides; NO, nitric oxide; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; Ccl2, C-C motif chemokine ligand 2; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-kappa B; IκBa, inhibitor of NF-κB α
The expression of C-C motif chemokine ligand 2 is induced through mitogen-activated protein kinases. IL-1 produced following nitric oxide synthase to the same extent as that of retinoids. Furthermore, 550 phage-like RAW264.7 cells.

Inflammatory factors by activated macrophages. Inflammation is an adaptive response that is triggered by noxious stimuli and conditions such as infection and tissue injury. As important inflammatory cells, macrophages are involved in initiating inflammatory responses. Upon activation by lipopolysaccharides, which constitute a component of the outer membrane of gram-negative bacteria, the production of numerous inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-6, and IL-1β is induced through mitogen-activated protein kinases (MAPK) and nuclear factor (NF)-κB pathways. Furthermore, nitric oxide (NO), a major inflammatory mediator, is produced following nitric oxide synthase (NOS) gene expression, which is involved in innate immunity as a toxic agent against infectious organisms. Monocyte chemoattractant protein-1 (MCP-1) is also produced through the expression of C-C motif chemokine ligand 2 (Ccl2), which regulates immune cell migration and infiltration into inflammation foci. Although these inflammatory factors contribute to host defenses against infectious organisms and to tissue homeostasis, the dysregulation of proinflammatory factor production can lead to many inflammatory disorders. Hence, to prevent inflammation-related diseases, it is crucial to regulate the overexpression of inflammatory factors by activated macrophages.

Full-length β-carotene has been reported to attenuate excessive inflammatory responses in murine macrophage-like RAW264.7 cells stimulated by LPS via inhibition of the NF-κB, JAK2/STAT3, and MAPK signaling pathways. However, the anti-inflammatory mechanisms of β-carotene-derived β-apocarotenoids against activated macrophages have not been established.

In the present study, we sought to prepare apo-β-carotene from β-carotene through chemical oxidation using potassium permanganate (KMnO4) and characterized the structure of seco-β-apo-8-carotenal by proton nuclear magnetic resonance (H-NMR) analysis for the first time. Furthermore, the inhibitory effects of this seco-type β-apocarotenoid against the overexpression of inflammatory mediators were investigated in LPS-activated macrophage-like RAW264.7 cells.

2 Experimental
2.1 Chemicals
Murine macrophage-like RAW264.7 cells were obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). RPMI 1640 medium, penicillin/streptomycin, and β-carotene were purchased from FUJIFILM Wako Pure Chemical Co., Ltd. (Osaka, Japan). Primary antibodies targeting phospho-SAPK/JNK 3 (JNK) (Thr183/Tyr185; rabbit polyclonal antibody), SAPK/JNK (rabbit polyclonal antibody), NF-κB p65 (D14E12; rabbit monoclonal antibody), and phospho-IκBα (Ser32/36; 5A5; mouse monoclonal antibody) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin (C4) sc-47778 (mouse monoclonal IgG) was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Anti-lamin B1 (mouse monoclonal) and anti-κBα (rabbit polyclonal) antibodies were purchased from Proteintech Group, Inc. (Rosemont, IL, USA). As secondary antibodies, goat anti-mouse and anti-rabbit IgG horseradish peroxidase antibodies were purchased from Abcam (Cambridge, UK). LPS from Escherichia coli, β-apo-8’-carotenal, squalanilamide, and N-(1-naphthyl) ethylenediamine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hexadecyltrimethylammonium bromide (CTAB) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Other chemicals and organic solvents were obtained from FUJIFILM Wako Pure Chemical Co., Ltd. (Osaka, Japan).

2.2 In vitro oxidation of β-carotene and chromatographic analysis
First, 5 mg of β-carotene and 0.8 mg of CTAB were dissolved in a solution of 40 mL chloroform and 10 mL KMnO4 solution (180 mg/10 mL distilled water). After 3 h of incubation at room temperature, 20 mL chloroform, 30 mL methanol, and 8 mL distilled water were added to the oxidation mixture, which separated into two phases. The chloroform phase (lower) was collected and dried using a rotary evaporator. Thin-layer chromatography (TLC) was performed using methanol on an RP-18 F254 plate (Merck Millipore, Burlington, MA, USA) to detect β-carotene oxidation products. The major β-carotene oxidation product (30 μg) was isolated by high-performance liquid chromatography (HPLC) under the following conditions, column: Devonil C30-UG-5 column (250 × 4.6 mm, Nomura Chemical Co., Aichi, Japan), mobile phase: methanol, flow rate: 1.0 mL/min, detection: 450 nm. Liquid chromatography-mass spectrometry (LC-MS) was performed to estimate the molecular ion mass of the purified oxidation product using LC-MS8040 (Shimadzu, Kyoto, Japan) with an ODS-UG-3 column (150 × 2.0 mm, Nomura Chemical Co., Aichi, Japan) and methanol as the mobile phase at a flow rate of 0.2 mL/min. The column temperature was maintained at...
30°C. A triple quadrupole mass spectrometer with electrospray ionization, in positive ion mode, was used for analyzing the total ion scanning range at m/z 50–700 under a nebulizer nitrogen gas flow rate of 2.0 L/min, drying nitrogen gas flow rate of 15.0 L/min, DL temperature of 250°C, and heat block temperature of 400°C.

2.3 Structural characterization of seco-β-apo-8'-carotenal by proton nuclear magnetic resonance (1H-NMR) analysis

1H-NMR spectra (500 MHz), including 1H-1H COSY and NOESY, were measured using a Varian UNITY INOVA 500 spectrometer in CDCl3.

2.4 Cell culture

RAW264.7 cells were cultured in RPMI 1640 with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere with 5% CO2 at 37°C. After pre-incubating RAW264.7 cells for 24 h, the culture medium was treated with carotenoids dissolved in dimethyl sulfoxide (DMSO) solution and incubated for an additional 2 h. Control cells were treated with DMSO alone. The final DMSO concentration was adjusted to 0.1% in the culture medium without cytotoxicity. Then, LPS (100 ng/mL) was added to the medium in the presence of carotenoids, and the cells were stimulated for an additional 30 min and 2 h (for western blotting), 6 h (for gene expression analysis), or 24 h (for NO, IL-6, IL-1β, and MCP-1 secretion analysis).

2.5 Cell viability

Cell viability was measured using WST-1 reagent. Briefly, RAW264.7 cells were treated with various concentrations of seco-β-apo-8'-carotenal for 24 h. After incubation, 10 µL of WST-1 was added to each well, and this was followed by incubation for 2 h. The absorbance of each well was determined at 450 nm using a microplate reader (Molecular Devices, CA, USA). Media samples with each seco-β-apo-8'-carotenal concentration without cells were prepared as blanks.

2.6 Determination of NO, IL-1β, and MCP-1 concentrations in culture media.

NO production was determined by the Griess method[20]. Culture media collected after LPS stimulation for 24 h were mixed equally with the Griess reagent (2.5% phosphoric acid, 1% sulfanilamide, and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water). The optical absorbance at 550 nm was measured, and the NO concentration was determined from a standard curve with NaNO3. Media containing carotenoids without cells were prepared as sample blanks. The levels of IL-6, IL-1β, and MCP-1 production in the culture medium were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Thermo Scientific, Frederick, MD, USA) according to the manufacturer’s protocol.

2.7 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from RAW264.7 cells using QIAzol lysis reagent (Qiagen, Hilden, Germany). Then, cDNA was synthesized from total RNA using ReverTra Ace (TOYOBO, Osaka, Japan) according to the manufacturer’s instructions. RT-qPCR analysis was performed with the StepOnePlus real-time PCR system (Applied Biosystems Japan Ltd, Tokyo, Japan). GeneAce Probe qPCR Mix II (Nippon gene, Tokyo, Japan) was used according to the manufacturer’s protocol with the following PCR cycling conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 30 s and 60°C for 1 min. mRNA expression levels were measured using TaqMan Gene Expression Assays (Thermo Fisher Scientific, Frederick, MD, USA) for Ccl2 (Mm00441242_m1), Il6 (Mm00446190_m1), Il1b (Mm00434228_m1), Rps18 (Mm02601777_g1), and Gapdh (Mm99999915_g1).

2.8 Western blotting

RAW264.7 cells were washed twice with ice-cold PBS and lysed with 50 µL ice-cold RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor). Cell lysates were centrifuged at 12,000 × g for 10 min at 4°C. Nuclear proteins were fractionated using the LysisPure Nuclear and Cytoplasmic Extractor Kit (FUJIFILM Wako Pure Chemical Co., Ltd., Osaka, Japan) according to the manufacturer’s protocol. Following centrifugation, the protein concentration in the supernatant was measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Then, proteins in the supernatant (10 µg of protein per lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with Tris-HCl buffer saline containing 1% Tween 20 (TBST) and 1% BSA for 1 h and then incubated with primary antibodies (JNK: 1:2500, phospho-JNK: 1:2000, NF-κB p65: 1:2500, IκBα: 1:5000, phospho-IκBα: 1:3000, β-actin: 1:3000, and lamin B1: 1:3000) at room temperature for 1 h. After washing three times with TBST, the membranes were incubated with secondary antibodies (1:20000) for 1 h at room temperature. Proteins were detected using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions.

2.9 Statistics

Data are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using one-way
3 Results

3.1 Identification of seco-type β-apocarotenoid in β-carotene oxidation products

Chemical oxidation of β-carotene (Fig. 2B) by KMnO₄ generated new TLC spots, although β-carotene itself no longer remained (Fig. 1A). We focused on the characteristic Peak 1 detected at 6.3 min by HPLC analysis (Fig. 1B), which showed a longer maximum absorption at 462 nm (Fig. 1B inset) and was a deeper orange (Rf 0.52, Fig. 1A) than the others. Peak 1 was the largest among the peaks at 450 nm and readily isolated due to clear separation (Fig. 1B). Furthermore, the retention time of Peak 1 was different from that of β-apo-8'-carotenal (22.8 min, Fig. 1B), which had an absorption maximum similar to that of Peak 1. After HPLC purification of Peak 1 (purity >99%, Fig. S1), the molecular ion was analyzed by LC-MS. The protonated ion at 449.4[M + H]⁺ and the sodium adduct ion at 471.4[M + Na]⁺ were predominantly observed, as shown in Fig. 1C. Subsequently, we analyzed molecular structures using NMR. The ¹H-NMR peaks were assigned based on the ¹H-NMR of β-carotene and NOESY experiments. This is the first report of the complete assignment of the ¹H-NMR of seco-β-apo-8'-carotenal (2E, 4E, 6E, 8E, 10E, 12E, 14E, 16E)-2, 6, 11, 15, 19, 19-hexamethyl-18, 23-dioxo-2, 4, 6, 8, 10, 12, 14, 16-tetracosaoctenal (Fig. 2A).

3.2 Cell viability of macrophages treated with seco-β-apo-8'-carotenal

Murine macrophage-like RAW264.7 cells were treated with seco-β-apo-8'-carotenal for 24 h, and their viability was measured using WST-1 assays. Cell viability was not influenced by 5 µM seco-β-apo-8'-carotenal treatment, although it decreased to 60% at 10 µM (Fig. 3). Therefore, seco-β-apo-8'-carotenal was administered at a concentration of 5 µM or less in the subsequent experiments.

![Analysis of β-carotene oxidation products by KMnO₄. A) Reverse-phase TLC chromatogram. Lane 1: β-carotene authentic standard (Rf 0.10). Lane 2: β-carotene oxidation mixture. The asterisk (Rf 0.52) represents the spot corresponding to Peak 1 in HPLC. B) Reverse-phase HPLC chromatogram of β-carotene oxidation products at 450 nm. Peak 1 showed a retention time of 6.3 min and maximum absorption at 462 nm (inset). C) MS spectrum of Peak 1.](image-url)
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3.3 Suppression of inflammatory mediators by seco-β-apo-8′-carotenal in LPS-activated RAW264.7 cells

We investigated the suppressive effects of seco-β-apo-8′-carotenal on inflammatory mediator production induced in LPS-activated RAW264.7 cells. Seco-β-apo-8′-carotenal significantly inhibited the secretion of inflammatory mediators and cytokines such as NO and MCP-1 (Table 2). Compared to the original β-carotene (Fig. 2B), seco-β-apo-8′-carotenal significantly decreased MCP-1 levels (Table 2), indicating that β-carotene oxidation augmented anti-inflammatory action against activated macrophages. To clarify the mechanism of anti-inflammatory action against LPS-activated RAW264.7 cells, the activity of seco-β-apo-8′-carotenal was compared with that of β-apo-8′-carotenal, which contains a β-ionone ring (Figs. 2A and 2C). Seco-β-apo-8′-carotenal significantly decreased the concentrations of IL-6, IL-1β, NO, and MCP-1 in the culture media of LPS-activated RAW264.7 cells (Figs. 4A-D). Furthermore, Il6, Il1b, and Ccl2 mRNA expression was downregulated in seco-β-apo-8′-carotenol-treated cells (Figs. 4A, 4C and 4D). These findings suggest that seco-β-apo-8′-carotenol suppressed the production of inflammatory factors by regulating gene expression. In contrast to seco-β-apo-8′-carotenol, β-apo-8′-carotenol did not inhibit the production of IL-6, NO, IL-1β, and MCP-1 (Figs. 4A-4D) or downregulate the expression of Il6, Il1b, and Ccl2 mRNAs (Figs. 4A, 4C and 4D). These results indicate that the opened β-ring structure of seco-β-apo-8′-carotenol plays a pivotal role in the anti-inflammatory action of activated RAW264.7 cells.

Table 1 ^1H-NMR analysis of the carotenoid generated by β-carotene oxidation.

| Position | δ (ppm) | mult. | J (Hz) |
|----------|---------|-------|--------|
| H₂-2     | ∼1.54 m | m     |        |
| H₂-3     | ∼1.49 m | m     |        |
| H₂-4     | 2.40 t (7) | t   |        |
| H-7      | 6.55 d (15) | d  |        |
| H-8      | 7.40 d (15) | d  |        |
| H-10     | 6.56 d (11) | d  |        |
| H-11     | 6.68 dd (15, 11) | dd |        |
| H-12     | 6.53 d (15) | d  |        |
| H-14     | 6.37 d (11) | d  |        |
| H-15     | 6.77 dd (15, 11) | dd |        |
| H₁-16    | 1.17 s  | s     |        |
| H₁-17    | 1.17 s  | s     |        |
| H₁-18    | 2.11 s  | s     |        |
| H₁-19    | 1.99 s  | s     |        |
| H₁-20    | 2.01 s  | s     |        |
| H-8′     | 9.46 s  | s     |        |
| H-10′    | 6.94 d (11) | d  |        |
| H-11′    | 6.70 dd (15, 11) | dd |        |
| H-12′    | 6.75 d (15) | d  |        |
| H-14′    | 6.45 d (11) | d  |        |
| H-15′    | 6.70 dd (15, 11) | dd |        |
| H₂-19′   | 1.91 s  | s     |        |
| H₂-20′   | 2.02 s  | s     |        |

m, multiplet; t, triplet; d, doublet; dd, doublet of doublets; s, singlet.

Fig. 2 Molecular structure of carotenoids used in this study. A) seco-β-apo-8′-carotenol, B) β-carotene, and C) β-apo-8′-carotenal.

Fig. 3 Cell viability of RAW264.7 cells treated with seco-β-apo-8′-carotenol. After treating cells with seco-β-apo-8′-carotenol at each concentration for 24 h, cell viability was determined by WST-1 assay. Data are mean ± SEM (n = 3). Bars with different letters are significantly different (p < 0.05). Statistical analysis was performed using one-way ANOVA followed by the Tukey-Kramer test.
3.4 Seco-β-apo-8′-carotenal inhibits protein phosphorylation in MAPK and NF-κB signaling pathways

To elucidate the anti-inflammatory mechanism of seco-β-apo-8′-carotenal, phosphorylated protein levels in the MAPK and NF-κB pathways associated with inflammatory signaling were investigated. As shown in Fig. 5A, the LPS-induced phosphorylation of JNK and the inhibitor of NF-κB α (IκBα) was diminished by seco-β-apo-8′-carotenal treatment. Furthermore, seco-β-apo-8′-carotenal significantly inhibited the nuclear accumulation of the transcription factor NF-κB p65 (Fig. 5B). These results indicate that seco-β-apo-8′-carotenal suppresses the mRNA expression of inflammatory factors by attenuating the MAPK and NF-κB signaling pathways.
4 Discussion

Apocarotenoids are widely found in plants, foods, and mammalian tissues. Previously, β-apocarotenoids have been reported to antagonize the activation of nuclear receptors, such as RAR, RXR, and PPAR, thereby inhibiting the agonist-induced expression of retinoid-responsive genes. However, the suppressive effect of β-apocarotenoids on inflammation remains unclear. Since chronic inflammation is associated with various diseases, we prepared a characteristic seco-type β-apocarotenoid via β-carotene oxidation and investigated its anti-inflammatory effects against activated macrophages.

Several studies have shown that β-carotene oxidation using KMnO₄ generates a series of β-apocarotenoids: β-apo-14'-carotenal, β-apo-12'-carotenal, β-apo-10'-carotenal, and β-apo-8'-carotenal. The reaction mechanism for the oxidative cleavage of β-carotene is thought to involve isomerization of an E- to Z- double bond and syn-addition of the permanganate ion, thus forming a cyclic permanganate ester, which is well established as an intermediate in this type of a reaction. These reactions give rise to the expected series of β-apocarotenals due to oxidative cleavage of the double bonds of the polyene backbone. Furthermore, β-carotene oxidation products generated by KMnO₄ also include certain seco-type carotenoids that result from the cleavage of the permanent Z-configured double bonds (C-5,6 and/or C-5',6' positions) in the β-ring. Here, the protonated ion at 449.4 [M + H]⁺ of Peak 1 (Fig. 1C) of the β-carotene oxidation products corresponded to that of seco-type β-apocarotenoid, as described by Gurak et al. Therefore, we identified for the first time the structure of Peak 1 as seco-β-apo-8'-carotenal by "H-NMR analysis, which was incompletely determined by Gurak et al. through NMR. At present, seco-β-apo-8'-carotenal is neither found in foods nor in mammalian tissues, although several β-apocarotenoids are detected through intake of carotenoid-containing foods and oxidative metabolism of β-carotene in vivo. On the other hand, there are several seco-type carotenoids including apo-bodies in edible bivalves such as freshwater clams and oysters; hence, these seco-carotenoids may be absorbed in the body. However, to the best of our knowledge, there is no information concerning the accumulation and distribution of seco-carotenoids in mammalian tissues. Thus, future studies are greatly needed for the investigation of seco-carotenoid metabolism.

To verify the anti-inflammatory effect of seco-β-apo-8'-carotenol, we examined the production of inflammatory mediators in LPS-activated RAW264.7 cells. Seco-β-apo-8'-carotenol significantly inhibited the overproduction of NO.
and MCP-1 (Table 2). Notably, seco-β-apo-8'-carotenal, but not the original β-carotene (Fig. 2B), markedly decreased MCP-1 secretion (Table 2), indicating that the oxidative cleavage of β-carotene augments its anti-inflammatory action.

Seco-β-apo-8'-carotenal significantly downregulated the mRNA expression of inflammatory factors such as Il6, Il1b, and Ccl2 (Fig. 4). In addition, seco-β-apo-8'-carotenal inhibited the phosphorylation of JNK and IkBα as well as the nuclear accumulation of NF-κB p65 (Fig. 5). Upon activation, JNK phosphorylates transcription factors such as c-Jun, c-Fos, and ATF. In turn, these factors constitute the activator protein-1 (AP-1) transcription factor, which regulates the expression of several stress-responsive genes, including inflammatory mediators and cytokines28, 29. Moreover, NF-κB activation converges on activating the IkB kinase (IKK) complex, leading to IkB phosphorylation and subsequent degradation. NF-κB released from IkB translocates to the nucleus to bind specific DNA sequences, thus activating the transcription of multiple genes, including inflammatory cytokines and chemokines28. Thus, seco-β-apo-8'-carotenal suppresses the secretion and gene expression of inflammatory factors by regulating the activation of MAPK and NF-κB signal cascades, thereby preventing excessive inflammation.

The present study found that the opened β-ring structure of seco-β-apo-8'-carotenal could play a pivotal role in anti-inflammatory action against activated macrophages (Fig. 4). Seco-β-apo-8'-carotenal significantly suppressed NO production and the secretion of IL-6, IL-1β, and MCP-1 by downregulating the mRNA expression of Il6, Il1b, and Ccl2, respectively (Fig. 4). In contrast, β-apo-8'-carotenol containing a β-ring did not inhibit pro-inflammatory factor production or gene expression (Fig. 4). As shown in Fig. 2, seco-β-apo-8'-carotenal introduces an α,β-unsaturated carbonyl group by opening the β-ring moiety of β-apo-8'-carotenal. Despite the presence of an α,β-unsaturated carbonyl group, β-apo-8'-carotenol fails to suppress inflammation (Fig. 4), indicating that β-apocarotenoids may require more than one α,β-unsaturated carbonyl group to induce anti-inflammatory action. Linnewiel-Hermoni et al.20 reported that lycopene derivatives containing two α,β-unsaturated carbonyl groups downregulate the mRNA levels of inflammatory factors such as TNF-α and MCP-1 in both cancer and bone cells. Regarding molecular mechanisms, the α,β-unsaturated carbonyl moiety of the lycopene derivatives directly interacted with the thiol groups of IKKβ, thereby attenuating IkB phosphorylation, which is followed by decreasing the nuclear translocation of NF-κB p6520. Interestingly, the molecular structures of these lycopene derivatives resemble the structure of seco-β-apo-8'-carotenol with two α,β-unsaturated carbonyl groups and a linear backbone. Seco-β-apo-8'-carotenol also significantly inhibited IkBα phosphorylation and NF-κB p65 accumulation in the nucleus (Fig. 5). Seco-β-apo-8'-carotenol may interact with intracellular proteins such as IKK, resulting in diminished inflammatory gene transcription.

In total lipid extracts of RAW264.7 cells treated with seco-β-apo-8'-carotenol for 26 h, several new peaks, as well as seco-β-apo-8'-carotenol were detected, although the molecular structures are unknown. In contrast, those peaks, except for seco-β-apo-8'-carotenol, were not detected in the culture medium after 26 h of incubation without cells. Thus, future work for the identification of seco-β-apo-8'-carotenol metabolites in cells is desired to reveal their anti-inflammatory mechanisms.

5 Conclusion

We successfully determined the molecular structure of seco-β-apo-8'-carotenol as a β-carotene-derived apocarotenoid. Seco-β-apo-8'-carotenol exhibited a characteristic structure in which one end-group formed an opened β-ring and the other was cleaved at the C-7',8' position of β-carotene. In LPS-activated murine macrophage-like RAW264.7 cells, seco-β-apo-8'-carotenol, but not β-apo-8'-carotenol, suppressed the overexpression of inflammatory factors by inhibiting the activation of the MAPK and NF-κB signaling pathways. Notably, the present results demonstrate that the anti-inflammatory action of seco-β-apo-8'-carotenol depends on the opened β-ring structure.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Supporting Information

This material is available free of charge via the Internet at http://dx.doi.org/ios.70.10.5650/jos.ess20329 (Figures of the HPLC chromatogram, 1H-NMR, COSY, and NOESY spectra of purified Peak 1 (seco-β-apo-8’-carotenol)).

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