

# Preparation of Apoastaxanthinals and Evaluation of Their Anti-inflammatory Action against Lipopolysaccharide-Stimulated Macrophages and Adipocytes

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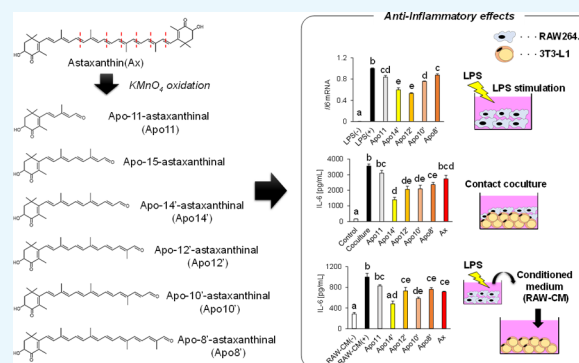


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**ABSTRACT:** Apocarotenoids are carotenoid derivatives in which the polyene chain is cleaved *via* enzymatic or nonenzymatic action. They are found in animal tissues and carotenoid-containing foods. However, limited information on the biological functions of apocarotenoids is available. Here, we prepared apocarotenoids from astaxanthin *via* chemical oxidation and evaluated their anti-inflammatory action against macrophages and adipocytes. A series of astaxanthin-derived apoastaxanthinals, apo-11-, apo-15-, apo-14'-, apo-12'-, apo-10'-, and apo-8'-astaxanthinals, were successfully characterized by chromatography and spectroscopic analysis. The apoastaxanthinals inhibited inflammatory cytokine production and mRNA expression against lipopolysaccharide-stimulated RAW 264.7 macrophages. Apoastaxanthinals suppressed interleukin-6 overexpression in an *in vitro* model with macrophages and adipocytes in the following cultures: (1) contact coculture of 3T3-L1 adipocytes and RAW264.7 macrophages and (2) 3T3-L1 adipocytes in a RAW264.7-derived conditioned media. These results indicate that the apoastaxanthinals have the potential for regulation of adipose tissue inflammation observed in obesity.



## 1. INTRODUCTION

Astaxanthin is a red carotenoid found in several marine animals and microorganisms.<sup>1</sup> It comprises a C<sub>40</sub> polyene skeleton with 13 conjugated double bonds and 2  $\beta$ -ionone rings substituted with hydroxy and keto groups. Previous reports indicate that astaxanthin possesses antioxidant and anticancer properties<sup>2,3</sup> and could be used for lifestyle-related disease prevention<sup>4</sup> and brain function improvement,<sup>5</sup> which has led to an increased interest in its applications in food, animal feed, and nutraceutical and pharmaceutical products.

Apocarotenoids are cleavage products formed through enzymatic or nonenzymatic reactions.<sup>6</sup> For example, retinoids, including vitamin A, are well-characterized apocarotenoids that play essential roles in many physiological processes including visual<sup>7</sup> and immune systems.<sup>8</sup> These compounds are produced through the central oxidative cleavage of provitamin A carotenoids by  $\beta$ -carotene 15,15'-oxygenase. In addition,  $\beta$ -carotene 9',10'-oxygenase (BCO2) which has been identified in mammals, can also recognize both non-provitamin A and provitamin A carotenoids as substrates and produce asymmetric apocarotenoids.<sup>9</sup>

It is established that  $\beta$ -carotene- and lycopene-derived apocarotenoids, including  $\beta$ -apo-10'-carotenal and apo-12'-lycopenal, are found in human plasma.<sup>10,11</sup> Furthermore, zeaxanthin- and lutein-derived apocarotenoids, including apo-

10'-zeaxanthinal and  $\epsilon$ -apo-12'-luteinal, were also identified in human plasma<sup>12</sup> and raw and processed foods.<sup>11,13</sup> However, only a few studies on astaxanthin-derived apocarotenoids, including 3-hydroxy-4-oxo- $\beta$ -ionone and 3-hydroxy-4-oxo- $\beta$ -ionol, were reported in human plasma.<sup>14</sup> Available information on the biological function of xanthophylls-derived apocarotenoids is also limited. On the other hand, it is known that  $\beta$ -carotene- and lycopene-derived apocarotenoids act as ligands for nuclear receptors including retinoid X receptor and retinoic acid receptor,<sup>15</sup> inhibiting the proliferation of some cancer cells such as leukemia and prostate cancer cell lines.<sup>16</sup> Further, apo-10'-lycopenoic acid inhibits liver and lung cancer cells by activating nuclear receptor peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ).<sup>17</sup>

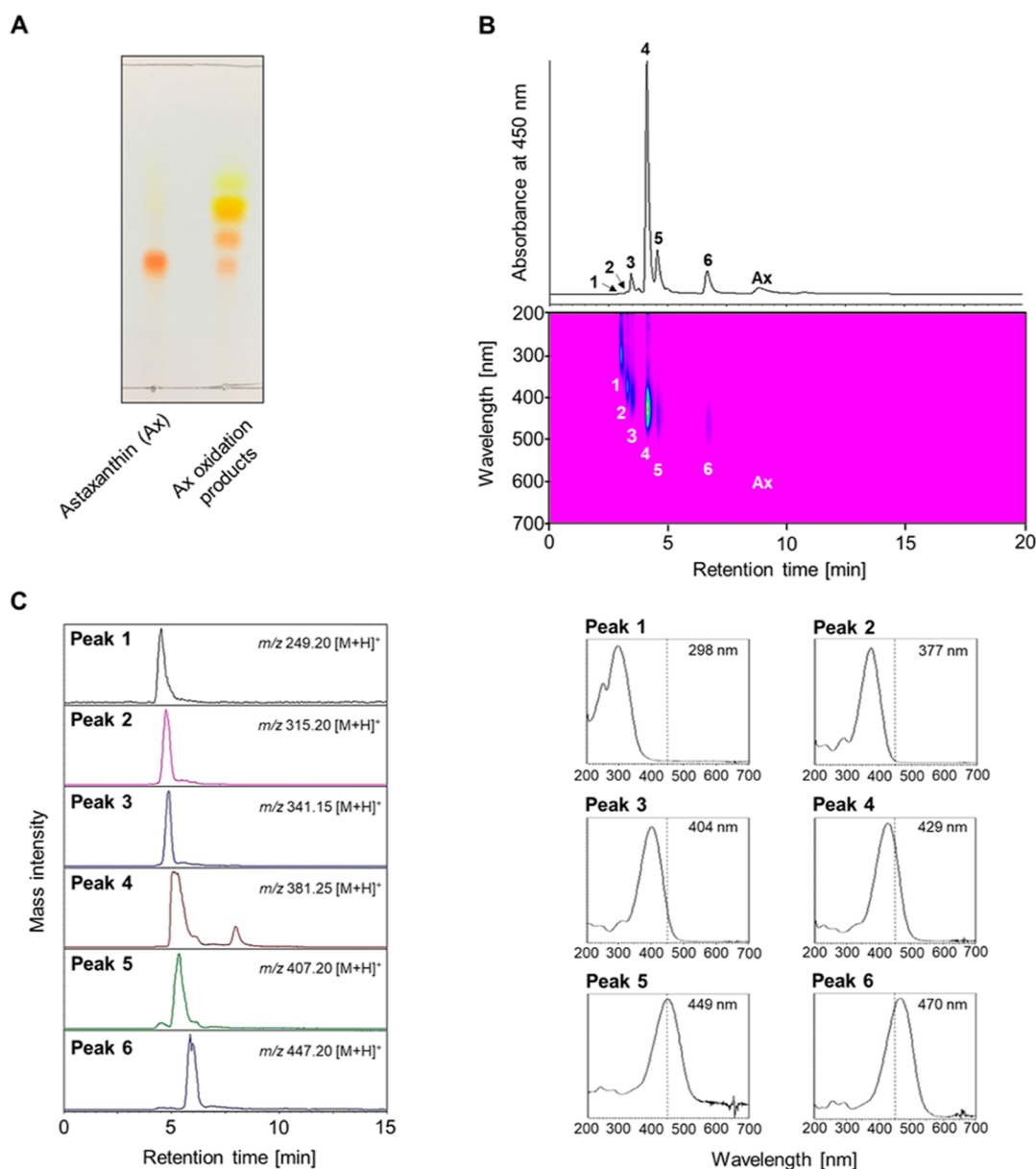
Inflammation is associated with the incidence and development of various noncommunicable diseases (NCDs) including fatty liver disease and diabetes mellitus.<sup>18</sup> Macrophages play an

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**Figure 1.** Identification of apoastaxanthinals generated by the oxidation of astaxanthin with potassium permanganate. (A) TLC analysis of astaxanthin and its oxidation products. (B) Reverse-phase HPLC equipped with C<sub>30</sub>-column was performed with methanol at a flow rate 1.0 mL/min. The products were monitored at wavelengths of 200 and 700 nm. Peak number described in the HPLC chromatogram corresponds to that on the photodiode array detector contour map (upper panel) and on the absorption spectra (lower panel). Absorption maxima are depicted within each figure, and black dotted lines are drawn at 450 nm. (C) LC-MS analysis equipped with C<sub>18</sub>-column was performed with methanol at a flow rate 0.1 mL/min. The peak number described in each mass chromatogram corresponds to that in (B).

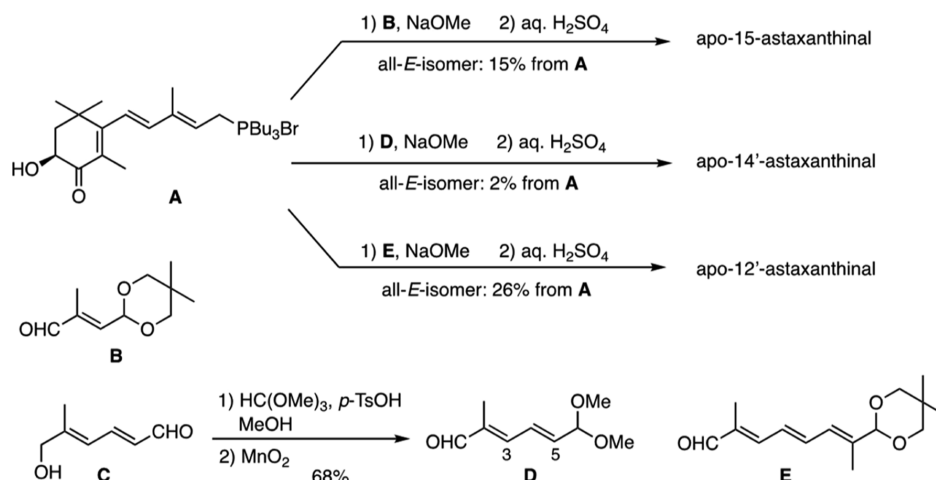
important role in regulating inflammation in the body.<sup>19</sup> They are responsible for the host's defense against infectious organisms and tissue homeostasis through the production of inflammatory cytokines and other mediators, including interleukin (IL), tumor necrosis factor- $\alpha$ , monocyte chemoattractant protein-1 (MCP-1), prostaglandins, and nitric oxide (NO).<sup>20,21</sup> The dysregulation of these mediators causes chronic inflammatory disorders, leading to NCDs.<sup>19</sup> In obese adipose tissues, activated macrophages stimulate adipocytes by secreting numerous inflammatory mediators. This exacerbates adipose tissue inflammation, which then leads to the development of insulin resistance and type-2 diabetes mellitus.<sup>22–25</sup> Hence, it is important to regulate the overproduction of inflammatory factors to prevent NCDs.

However, there is little information regarding regulatory effects of apocarotenoids against inflammation.

It was previously reported that astaxanthin inhibited NO and prostaglandin E<sub>2</sub> by downregulating *Nos2* and *Ptgs2* mRNA expression in lipopolysaccharides (LPS)-activated RAW264.7 macrophages.<sup>26</sup> Furthermore, it was demonstrated that astaxanthin suppressed inflammation in the adipose tissue of diet-induced obesity mouse model.<sup>27</sup> However, the anti-inflammatory activity of astaxanthin-derived apocarotenoids has not been established.

In this study, we prepared astaxanthin-derived apocarotenoids through the oxidation of astaxanthin with potassium permanganate (KMnO<sub>4</sub>). The anti-inflammatory activity of the apoastaxanthinals was then investigated in activated RAW264.7

## Scheme 1. Synthesis of Apo-15'-astaxanthinal, Apo-14'-astaxanthinal, and Apo-12'-astaxanthinal



macrophages. In addition, using an obesity-induced inflammation *in vitro* model, we evaluated the inhibitory effect of apoastaxanthinals against inflammatory cytokine production induced through the interaction between 3T3-L1 adipocytes and RAW264.7 macrophages. This study demonstrated that the cleavage products derived from astaxanthin have a potential for prevention of inflammation-related NCDs.

## 2. RESULTS

**2.1. Preparation and Identification of Apocarotenoids Generated by Chemical Oxidation of Astaxanthin.** From the oxidation of astaxanthin with  $\text{KMnO}_4$ , new spots of various colors were observed on the thin-layer chromatography (TLC) plate (Figure 1A). Subsequently, reverse-phase high-performance liquid chromatography (HPLC) equipped with a  $\text{C}_{30}$  column successfully separated astaxanthin and the six derivatives that had earlier retention time and shorter absorption maxima than the parent astaxanthin (Figure 1B). Furthermore, liquid chromatography–mass spectrometry (LC–MS) analysis revealed that these peaks had smaller molecular ions (protonated ions of peak 1–6;  $m/z$  249.20, 315.20, 341.15, 381.25, 407.20, and 447.20) than astaxanthin (Figure 1C), indicating that the astaxanthin oxidation products had shorter chain lengths. To compare the oxidation products, we synthesized apoastaxanthinals (Scheme 1 and Supporting Information). According to the corresponding HPLC retention times and absorption spectra, peaks 2, 3, and 4 were identified as apo-15'-astaxanthinal, apo-14'-astaxanthinal (apo14'), and apo-12'-apoastaxanthinal (apo12'), respectively. Peaks 1, 5, and 6 were further purified using HPLC and characterized by  $^1\text{H}$  NMR analysis (Table 1). The UV–vis, molecular ion, and  $^1\text{H}$  NMR data of peaks 1, 5, and 6 were identical to the previously reported data for apo-11'-astaxanthinal (apo11), apo-10'-astaxanthinal (apo10'), and apo-8'-astaxanthinal (apo8'), respectively.<sup>28</sup> Thus, six apoastaxanthinals were prepared by the oxidation of astaxanthin with  $\text{KMnO}_4$  (Figure 2).

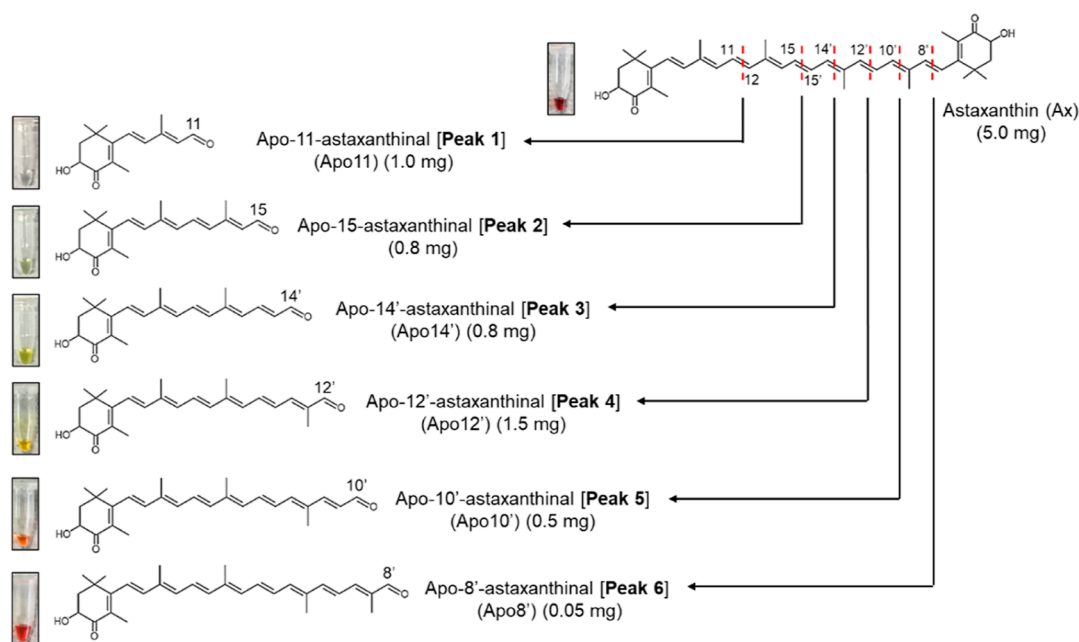
**2.2. Cell Viability of RAW264.7 and 3T3-L1 Cells Treated with Apoastaxanthinals and Astaxanthin.** To evaluate the cell viability by WST-1 assay, RAW264.7 or differentiated 3T3-L1 cells were incubated in the presence of apoastaxanthinals or astaxanthin for 24 h. It was observed that the viability of the RAW264.7 and 3T3-L1 cells was not affected by the treatment (5  $\mu\text{M}$ ) with carotenoids (Figure 3).

**Table 1.**  $^1\text{H}$  NMR Analysis of Peaks 1, 5, and 6 Generated by Astaxanthin Oxidation<sup>a</sup>

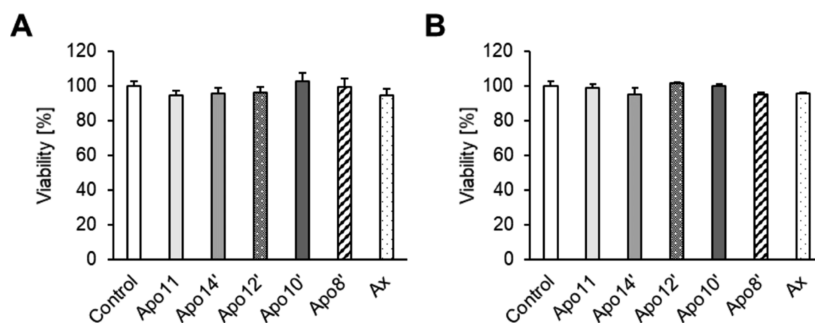
position	peak 1		peak 5		peak 6	
	$\delta$ (ppm)	mult. J (Hz)	$\delta$ (ppm)	mult. J (Hz)	$\delta$ (ppm)	mult. J (Hz)
H-2 $\alpha$	2.2	dd (13, 5)	2.18	dd (13, 5)	2.16	dd (13.5, 6)
H-2 $\beta$	1.88	dd (13, 13)	1.84	dd (13, 13)	1.82	dd (13.5, 13.5)
H-3	4.35	ddd (13, 5, 2)	4.35	ddd (13, 5, 2)	4.33	ddd (13.5, 6, 2)
H-7	6.7	d (17)	6.27	d (17)	6.23	d (15.5)
H-8	6.36	d (17)	6.45	d (17)	6.43	d (15.5)
H-10	6.03	d (7)	6.32	d (11)	6.3	d (11)
H-11	10.18	d (7)	6.76	dd (15, 11)	6.68	dd (15, 11)
H-12			6.49	d (15)	6.45	d (15)
H-14			6.36	d (11)	6.33	d (11)
H-15			6.88	dd (15, 11)	6.77	dd (15, 11)
H <sub>3</sub> -16	1.34	s	1.35	s	1.33	s
H <sub>3</sub> -17	1.19	s	1.23	s	1.21	s
H <sub>3</sub> -18	1.89	s	1.96	s	1.95	s
H <sub>3</sub> -19	2.35	s	2.04	s	2.02	s
H <sub>3</sub> -20			2.05	s	2.02	s
H-8'					9.46	s
H-10'			9.62	d (9)	6.94	d (11)
H-11'			6.23	dd (15, 9)	6.69	dd (15, 11)
H-12'			7.18	d (15)	6.75	d (15)
H-14'			6.64	d (11)	6.45	d (11)
H-15'			6.68	dd (15, 11)	6.7	dd (15, 11)
H <sub>3</sub> -19'					1.91	s
H <sub>3</sub> -20'			1.94	s	2.02	s
OH	3.68	d (2)	3.4	d (2)	3.68	d (2)

<sup>a</sup>s, singlet; d, doublet; dd, doublet-of-doublets; ddd, doublet-of-doublets-of-doublets.

**2.3. Inhibitory Effect of Apo-12'-astaxanthinal on the Inflammatory Factor Expression in Activated RAW264.7 Macrophages.** Anti-inflammatory activity of apo-12'-astaxanthinal was evaluated using LPS-activated RAW264.7 cells. In the culture media of the apo12'-treated cells, lower levels of inflammatory factors were observed than those in the LPS-treated [LPS (+)] and LPS and astaxanthin-treated (Ax) groups (Figure 4A). Further, treatment of apo12' significantly downregulated mRNA expression of *Il6*, *Il1b*, *Nos2*, and *Ccl2* than LPS (+) and Ax groups (Figure 4B). These results



**Figure 2.** Structure of the apoastaxanthins derived from astaxanthin. The number between the brackets after each compound name corresponds to each peak described in Figure 1. The amount of astaxanthin used as a starting material and the yields of the six products are stated in milligrams. Pictures depict each apoastaxanthinal and astaxanthin dissolved in DMSO.



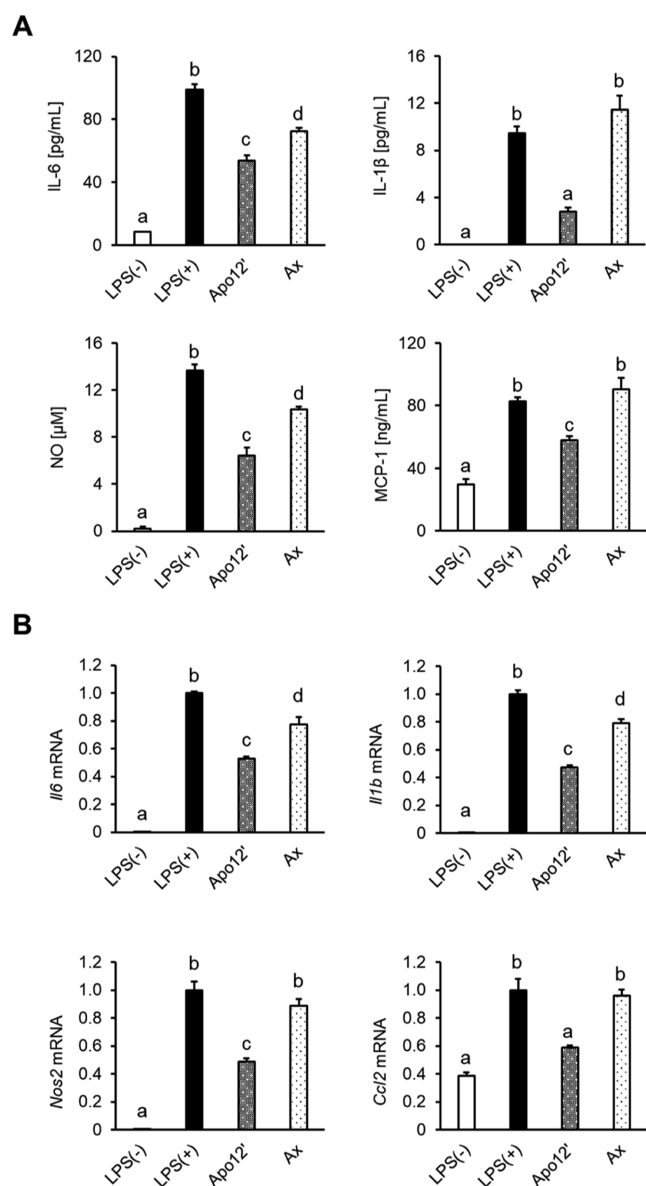
**Figure 3.** Cell viability of the macrophage-like RAW264.7 cells (A) and differentiated 3T3-L1 adipocytes (B) treated with apoastaxanthins and astaxanthin. After incubation with each carotenoid ( $5 \mu\text{M}$  for 24 h), cell viability was determined using the WST-1 assay. Data are represented as the mean  $\pm$  SEM [ $n = 5$  (A) or  $n = 3$  (B)]. Statistical analysis was performed using one-way ANOVA followed by the Tukey's HSD test. Apo11: apo-11-astaxanthinal. Apo14': apo-14'-astaxanthinal. Apo12': apo-12'-astaxanthinal. Apo10': apo-10'-astaxanthinal. Apo8': apo-8'-astaxanthinal. Ax: astaxanthin.

indicate that apo-12'-astaxanthinal is an effective anti-inflammatory agent against LPS-stimulated macrophages.

**2.4. Downregulation of the Inflammatory Factor Gene Expression in Activated RAW264.7 Macrophages Treated with Apoastaxanthins.** The anti-inflammatory activities of the apoastaxanthins were compared in activated RAW264.7 cells. It was observed that the mRNA levels of *Il6*, *Il1b*, and *Ptgs2* were downregulated by the treatment with the apoastaxanthinal derivatives, although those of *Nos2* were not changed in the apo10'-treated cells (Figure 5). Among the apoastaxanthinal derivatives, apo14' and apo12' strongly downregulated the inflammatory factor mRNA expression. By contrast, the suppressive effects were comparable between apo11, apo10', and apo8' (Figure 5). These results indicated that the apoastaxanthinal derivatives downregulated the inflammatory factor mRNA expression in LPS-activated RAW264.7 and that the anti-inflammatory activity was dependent on their chain length.

**2.5. Apoastaxanthins Inhibited IL-6 Production and mRNA Expression Induced by Interaction between Macrophages and Adipocytes.** Dysregulation of macrophage and adipocyte interactions causes obesity-induced inflammation followed by insulin resistance. To ensure the suppressive effect against this interaction *in vitro*, we cocultured RAW264.7 with differentiated 3T3-L1 cells in the presence of apoastaxanthins (Figure 6A). Compared to those in the coculture group, IL-6 protein levels in the culture media were reduced after treatment with the apoastaxanthins, except for apo11' (Figure 6A upper panel). In addition, *Il6* mRNA expression was downregulated in apo12'- and apo10'-treated cells. In particular, its expression was the lowest in the apo14'-treated cells (Figure 6A lower panel). These results suggest that apoastaxanthins attenuated IL-6 production induced by the coculture of 3T3-L1 adipocytes and RAW264.7 macrophages by downregulating its mRNA expression.

To investigate the anti-inflammatory action of the apoastaxanthins against 3T3-L1 adipocytes, the cells were



**Figure 4.** Apo-12'-astaxanthin significantly inhibited the expression of anti-inflammatory factors compared with parental astaxanthin in activated RAW264.7 macrophages. (A) Carotenoid-pretreated ( $5 \mu\text{M}$  for 2 h) cells are stimulated by LPS (100 ng/mL for 24 h) in the presence of carotenoids ( $5 \mu\text{M}$ ). The levels of each inflammatory factor in the culture supernatant were determined by ELISA and Griess method. (B) Carotenoid-pretreated ( $5 \mu\text{M}$  for 2 h) cells are stimulated by LPS (100 ng/mL for 6 h). The levels of each inflammatory factor mRNA expression were determined by quantitative PCR method. *Gapdh* was used as the endogenous control. Data are represented as the mean  $\pm$  SEM ( $n = 3$ ) with different letters ( $p < 0.05$ ). Apo12': apo-12'-astaxanthin. Ax: astaxanthin.

incubated in RAW-CM recovered from the RAW264.7 cultures (Figure 6B). Although incubation with RAW-CM(+) significantly increased the IL-6 levels compared to that with RAW-CM(-), the addition of astaxanthin and apoastaxanthins, except for apo11, significantly decreased the IL-6 levels (Figure 6B upper panel). In addition, apo14', apo12', and apo10' significantly downregulated *Il6* mRNA expression induced by RAW-CM(+) in 3T3-L1 cells (Figure 6B, lower panel). Notably, apo14' attenuated both IL-6 protein and mRNA

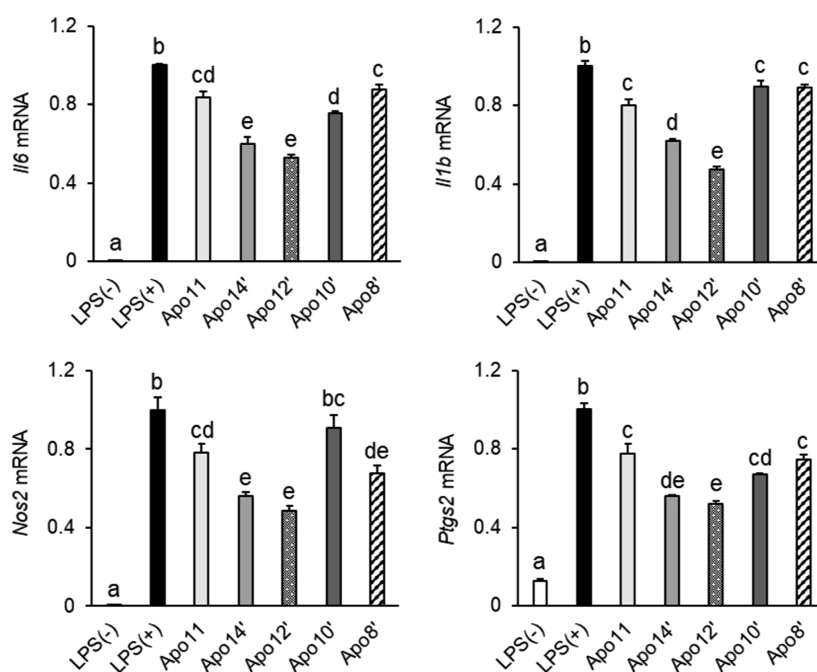
expression to the same level as that with RAW-CM(-) (Figure 6B). These results indicate that apoastaxanthins, especially apo14', can regulate IL-6 expression, which is induced by humoral factors secreted from LPS-stimulated RAW264.7 cells.

### 3. DISCUSSION

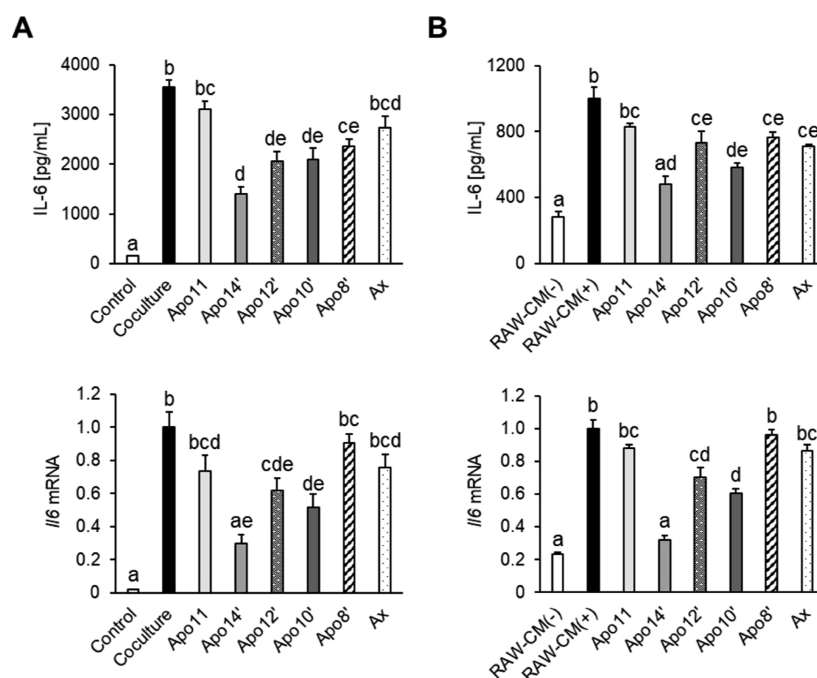
It is established that the auto-oxidation of astaxanthin generates several carotenoids.<sup>28</sup> To date, however, there have been few investigations on the preparation and biological activity of apoastaxanthin derivatives. In this study, through the oxidation of astaxanthin with  $\text{KMnO}_4$ , we synthesized and characterized six apoastaxanthin derivatives, namely, apo-11-, apo-10', apo-8', apo-15-, apo-14', and apo-12'-apoastaxanthin. We also evaluated their anti-inflammatory actions against activated RAW264.7 and 3T3-L1 cells. Although the reaction of astaxanthin with peroxynitrite generates apo-12'- and apo-10'-astaxanthin,<sup>29</sup> to our knowledge, this is the first report of apoastaxanthin synthesis *via* astaxanthin oxidation with  $\text{KMnO}_4$  (Figure 2).

Analysis of the anti-inflammatory activity of the apoastaxanthin derivatives on LPS-stimulated RAW264.7 cells revealed that apo12' significantly inhibited the production of IL-6, MCP-1, IL-1 $\beta$ , and NO *via* downregulating their mRNA expression (Figure 4). Compared with astaxanthin, apo12' potently suppressed these inflammatory factor expressions, suggesting that the cleavage products derived from astaxanthin augmented the anti-inflammatory activity on the LPS-stimulated macrophages (Figure 4). Notably, the downregulation of inflammatory factor gene expression was different among the apoastaxanthin derivatives with different chain lengths (apo11, apo14', apo12', apo10', and apo8'). Interestingly, apo14' (the number of carbons; C22) and apo12' (C25) displayed stronger suppressive properties compared with apo10' (C27), apo8' (C30), and apo11 (C15) in activated RAW264.7 cells (Figure 5). Apo-12'-lycopenal (C25), unlike apo-6' (C32) and apo-8'-lycopenal (C30), specifically activated transcription mediated by PPAR $\gamma$ .<sup>30</sup> PPAR $\gamma$  activation attenuated LPS-induced inflammation in RAW264.7 cells.<sup>31</sup> Thus, the data obtained in this study suggest that the chain length of the apoastaxanthin derivatives should be crucial for the anti-inflammatory effects.

Excessive and chronic production of inflammatory factors such as IL-6 from adipocytes is known to induce insulin resistance.<sup>24</sup> Therefore, we investigated the inhibitory action of apoastaxanthins against IL-6 production using two types of cell culture system: (1) the contact coculture of 3T3-L1 and RAW264.7 cells and (2) 3T3-L1 adipocytes in RAW-CM. The purpose of cell culture system 1 was to evaluate the regulatory effect on the direct interaction between macrophages and adipocytes. However, this system is unable to distinguish the effects of apoastaxanthins on each cell. Therefore, we then perform cell culture system 2 to investigate the effect of apoastaxanthins on adipocytes stimulated by macrophage-derived humoral factors associated with obesity-induced inflammation. Although the contact coculture demonstrated an increase in IL-6 production compared to the control group (RAW264.7 and 3T3-L1 alone), the apoastaxanthins, except for apo11, significantly attenuated IL-6 production (Figure 6A). Apo14' was the most potent at inhibiting IL-6 production by downregulating *Il6* mRNA expression (Figure 6A). Further, mRNA expression of other inflammatory cytokines such as *Il1b* and *Ccl2* were downregulated by apo14' treatment (Supporting Information). On the other hand, RAW-CM(+) promoted



**Figure 5.** Apoastaxanthins suppressed the mRNA expression of inflammatory factors in the activated RAW264.7 macrophages. Carotenoid-pretreated ( $5 \mu\text{M}$  for 2 h) cells are stimulated by LPS (100 ng/mL for 6 h) in the presence of carotenoids ( $5 \mu\text{M}$ ). The levels of each inflammatory factor mRNA expression were determined by the quantitative PCR method. *Gapdh* was used as the endogenous control. Data are represented as the mean  $\pm$  SEM ( $n = 3$ ) with different letters ( $p < 0.05$ ). Apo11: apo-11-astaxanthinal. Apo14': apo-14'-astaxanthinal. Apo12': apo-12'-astaxanthinal. Apo10': apo-10'-astaxanthinal. Apo8': apo-8'-astaxanthinal.



**Figure 6.** Apoastaxanthins and astaxanthin suppressed IL-6 production and mRNA expression induced by the interaction between RAW264.7 macrophages and 3T3-L1 adipocytes. (A) Contact coculture of RAW264.7 and 3T3-L1 cells (day 10) in the presence or absence of carotenoids ( $5 \mu\text{M}$  for 24 h). (B) Incubation of 3T3-L1 adipocytes in RAW264.7-derived conditioned media (RAW-CM). 3T3-L1 cells (day 10) were incubated in RAW-CM in the presence or absence of carotenoids ( $5 \mu\text{M}$  for 24 h). RAW-CM(-) represents the media collected from RAW264.7 cultured without LPS. Secreted IL-6 protein in the culture supernatant and *Il6* mRNA levels were evaluated by ELISA and quantitative PCR methods, respectively. *Actb* was used as the endogenous control. Data are represented as the mean  $\pm$  SEM ( $n = 3$ ) with different letters ( $p < 0.05$ ). Apo11: apo-11-astaxanthinal. Apo14': apo-14'-astaxanthinal. Apo12': apo-12'-astaxanthinal. Apo10': apo-10'-astaxanthinal. Apo8': apo-8'-astaxanthinal. Ax: astaxanthin.

the production of IL-6 in the 3T3-L1 cells compared to RAW-CM(-). This indicates that the activated macrophages also

exacerbated inflammation in the adipocytes through the secretion of inflammatory humoral factors (Figure 6B). We

also observed that astaxanthin and apoastaxanthins, except for apo11, suppressed the IL-6 production in 3T3-L1 cells induced by RAW-CM(+). Notably, apo14' strongly suppressed both the IL-6 production and mRNA expression when compared with other derivatives and astaxanthin. These results indicate that apo14' significantly attenuated the inflammatory reaction in 3T3-L1 cells in addition to RAW264.7 cells, suggesting that the anti-inflammatory action of apoastaxanthins requires a specific chain length.

Anti-inflammatory activity of apo14' and apo12' looks similar in LPS-stimulated RAW264.7 cells (Figure 5). However, apo14' showed much more potent anti-inflammatory activity in systems using RAW264.7 and 3T3-L1 cells (Figure 6). This difference may be caused by PPAR $\gamma$  activation by the apoastaxanthins on each cell. In addition to RAW264.7 macrophages,<sup>31</sup> inflammation of 3T3-L1 adipocytes is suppressed by activation of PPAR $\gamma$ .<sup>32</sup> Since adipocytes express more PPAR $\gamma$  proteins and genes than macrophages,<sup>33</sup> apo14' can exhibit potent anti-inflammatory activity in systems using RAW264.7 and 3T3-L1 cells. On the other hand, antioxidant enzymes associate with anti-inflammatory action by carotenoids.<sup>34</sup> Lycopene-derived apocarotenoids strongly upregulate the gene and protein expression of antioxidant enzymes such as heme oxygenase 1 and NAD(P)H:quinone oxidoreductase 1 than lycopene.<sup>35</sup> These enzyme expressions may be influenced by the treatment of apoastaxanthins and astaxanthin in each cell. Since astaxanthin treatment did not increase the intracellular levels of apoastaxanthins in both RAW264.7 and 3T3-L1 cells (data not shown), astaxanthin and apoastaxanthins can exhibit anti-inflammatory effects *via* different mechanisms. Future work for the understanding of anti-inflammatory mechanism of apoastaxanthins is expected.

Interestingly, apo10' also exerted a potent anti-inflammatory activity than parent astaxanthin (Figure 6). Since BCO2 can produce apo-10'-carotenoids from parent xanthophylls,<sup>9</sup> astaxanthin may be metabolized to apo10' in the body. Recently, it has been reported that astaxanthin is accumulated in the liver of BCO2 knockout mouse fed astaxanthin,<sup>36</sup> suggesting that astaxanthin is metabolized by BCO2 in the body. Given that astaxanthin suppresses inflammation in the adipose tissue of diet-induced obesity mouse model,<sup>27</sup> apo10' may be associated with this effect. Further investigation for the anti-inflammatory mechanism, metabolism, and distribution of apoastaxanthins in the body is needed. To elucidate them, this study demonstrating the preparation of apoastaxanthins and biological activity can be helpful.

## 4. CONCLUSIONS

We prepared and characterized six apoastaxanthins from astaxanthin with KMnO<sub>4</sub>, namely, apo-11-, apo-15-, apo-14', apo-12', apo-10', and apo-8'-astaxanthins. These apoastaxanthins inhibited the LPS-induced mRNA expression of inflammatory cytokines and mediators in RAW264.7 macrophages. Furthermore, the apoastaxanthins suppressed the overexpression of inflammatory cytokine IL-6 induced by the interaction of RAW264.7 and differentiated 3T3-L1 cells in an obesity-induced inflammation *in vitro* model. These results indicate, for the first time, the potential of astaxanthin-derived apoastaxanthins as health beneficial compounds.

## 5. EXPERIMENTAL SECTION

**5.1. Reagents and Chemicals.** RAW264.7 macrophages and 3T3-L1 preadipocytes were purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK) and the American Type Culture Collection (Manassas, VA, USA), respectively. Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). LPS from *Escherichia coli* O111:B4, isobutylmethylxanthine (IBMX), dexamethasone, insulin, *N*-[1-naphthyl]ethylenediamine dihydrochloride, and sulfanilamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Cetyltrimethylammonium bromide (CTAB) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Penicillin/streptomycin, RPMI1640, astaxanthin, and organic solvents were purchased from Fujifilm Wako Pure Chemical Co. Ltd. (Osaka, Japan).

**5.2. Chemical Oxidation of Astaxanthin and Chromatography Analysis.** Astaxanthin (5 mg) and CTAB (0.8 mg) were dissolved in 40 mL of chloroform, and then 10 mL of KMnO<sub>4</sub> solution was added (180 mg/10 mL in distilled water). After 3 h of reaction at room temperature (20–25 °C), the oxidation products were separated using chloroform/methanol/distilled water (10:5:3, v/v/v). The organic layer was then collected, and the solvent was removed *in vacuo*. Astaxanthin oxidation was confirmed by TLC with RP-18 F<sub>254S</sub> plates (Merck Millipore, Burlington, MA, USA), performed using methanol. LC–MS was carried out using an LCMS-8040 (Shimadzu, Kyoto, Japan) spectrometer with an ODS-UG-3 (150 × 2.0 mm, Nomura Chemical Co., Inc., Aichi, Japan) column. The column temperature was set at 30 °C, and methanol was eluted as the mobile phase at a flow rate of 0.1 mL/min. A triple quadrupole mass spectrometer with electrospray ionization (positive ion mode) was used with a total ion scanning range of *m/z* 50–700 under the following conditions: nebulizer gas (N<sub>2</sub>, 2.0 L/min), drying gas (N<sub>2</sub>, 15.0 L/min), desolvation line temperature (250 °C), and heat block temperature (400 °C). Isolation of each astaxanthin oxidation product was conducted by HPLC with an SPD-M20A detector (Shimadzu) and a C30-UG-5 column (250 × 4.6 mm, Nomura Chemical Co.) eluted by methanol (1.0 mL/min flow rate).

**5.3. Identification of Apo-11-astaxanthinal, Apo-10'-astaxanthinal, and Apo-8'-astaxanthinal Using <sup>1</sup>H NMR Analysis.** <sup>1</sup>H NMR (500 MHz) analysis, including <sup>1</sup>H–<sup>1</sup>H COSY and NOESY, were conducted using a Varian UNITY INOVA 500 spectrometer in CDCl<sub>3</sub>.

**5.4. Synthesis of Apo-15-astaxanthinal, Apo-14'-astaxanthinal, and Apo-12'-astaxanthinal.** These apoastaxanthins were chemically synthesized by Wittig condensation of previously reported phosphonium salt A<sup>37</sup> with acetal-aldehydes B,<sup>38</sup> D, and E<sup>39</sup> and subsequent acid-hydrolysis as shown in Scheme 1. Acetal-aldehyde D was prepared by dimethylacetalization of hydroxy-aldehyde C<sup>40</sup> and subsequent MnO<sub>2</sub>-oxidation. Experimental details are described in the Supporting Information.

**5.5. Cell Culture.** All cells used in this study were incubated in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. RAW264.7 cells (passage number 15–25) were cultured in RPMI 1640 with 10% FBS containing 100 μg/mL streptomycin and 100 U/mL penicillin. Twenty-four hours preincubated RAW264.7 cells were treated with carotenoids (5 μM) dissolved in dimethyl sulfoxide (DMSO) and incubated

for an additional 2 h. Control groups were treated with DMSO alone. To prevent cytotoxicity, DMSO was added to all culture media to 0.1%. LPS (100 ng/mL) was then added to the media, and the cells were stimulated for an additional 6 h (for mRNA expression analysis) or 24 h (for MCP-1, IL-6, IL-1 $\beta$ , and NO secretion analysis).

3T3-L1 preadipocytes (passage number 4) were cultured in DMEM with 10% FBS containing 100  $\mu$ g/mL streptomycin and 100 U/mL penicillin. After reaching confluence (day 0), 3T3-L1 cells were incubated in fresh DMEM for another 2 days (day 0–2). To differentiate, the cells were replaced in fresh DMEM with 1  $\mu$ M dexamethasone, 500  $\mu$ M IBMX, and 10  $\mu$ g/mL insulin and incubated for 2 days (day 2–4). On day 4, the media were replaced to fresh DMEM with 5  $\mu$ g/mL insulin. The insulin-containing media were changed every 2 days. On day 10, the differentiated 3T3-L1 cells were used for further experiments (the picture of differentiated 3T3-L1 cells at day 10 in the [Supporting Information](#)).

**5.6. Viability of RAW264.7 and 3T3-L1 Cells.** RAW264.7 macrophages ( $2 \times 10^4$  cells/well) or differentiated 3T3-L1 cells were inoculated to 96-well culture plates and then treated with 5  $\mu$ M of each carotenoid for 24 h. Then, WST-1 reagent (10  $\mu$ L of each well) was added and incubated for an additional 4 h. The absorbance at 450 nm of each well was determined using a microplate reader (Molecular Devices, CA, USA). Media samples containing each carotenoid without cells were used as blanks.

**5.7. Coculture of RAW264.7 and 3T3-L1 Cells.** As previously described,<sup>23</sup> RAW264.7 cells ( $1 \times 10^5$  cells/mL) were inoculated onto 3T3-L1 cells and incubated for an additional 24 h in the presence or absence of carotenoids without insulin. As a control, each cell, the number of which were equal to those in the contact system, was cultured separately and mixed after harvesting. The control group was treated with DMSO alone. To prevent cytotoxicity, DMSO was added to all culture media to 0.1%. Using a commercially available kit (Thermo Fisher Scientific, Frederick, MD, USA), the supernatant was subjected to enzyme-linked immunosorbent assay (ELISA). The adherent cells after removing the culture supernatant were subjected to mRNA expression analysis.

**5.8. RAW-CM Preparation and Stimulation to Differentiated 3T3-L1 Adipocytes.** RAW264.7-derived conditioned media (RAW-CM) were prepared following previous reports<sup>22,41</sup> with a few modifications. In brief, RAW264.7 cells ( $5 \times 10^4$  cells/mL) in DMEM with 10% FBS were preincubated in 24-well plates for 48 h. The media was then replaced with DMEM with or without LPS (100 ng/mL). After 12 h of stimulation, the cells were then incubated in DMEM without LPS for an additional 12 h. The culture media were filtered using Millex-GP 0.22  $\mu$ m (Merck Millipore, Burlington, MA, USA) and stored at  $-80$   $^{\circ}$ C for further experiments (RAW-CM). The CM recovered from RAW264.7 cells treated with or without LPS was named RAW-CM(+) and RAW-CM(–), respectively. 3T3-L1 adipocytes were incubated in RAW-CM in the presence or absence of carotenoids for 24 h without insulin. The cells in the RAW-CM(–) group were treated with DMSO alone. To prevent cytotoxicity, DMSO was added to all culture media to 0.1%. After 24 h incubation in RAW-CM, the culture supernatant and the cells were subjected to ELISA and mRNA expression analysis, respectively.

**5.9. Reverse Transcription Quantitative PCR.** Total RNA was obtained using the QIAzol lysis reagent (Qiagen, Hilden, Germany). To synthesize cDNA from the total RNA, reverse transcription was performed using ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's protocol. Gene expression level was quantified using GeneAmp Probe qPCR Mix II (Nippon gene, Tokyo, Japan) on the StepOnePlus real-time PCR system (Applied Biosystems Japan Ltd., Tokyo, Japan). The PCR cycling condition was 50  $^{\circ}$ C for 2 min, 95  $^{\circ}$ C for 10 min, and 40 cycles of 95  $^{\circ}$ C for 30 s and 60  $^{\circ}$ C for 1 min. TaqMan Gene Expression Assays purchased from Thermo Fisher Scientific were follows: *Nos2* (Mm00440502\_m1), *Il6* (Mm00446190\_m1), *Ccl2* (Mm00441242\_m1), *Ptgs2* (Mm00478374\_m1), *Il1b* (Mm00434228\_m1), *Gapdh* (Mm99999915\_g1), and *Actb* (Mm00607939\_s1). Relative quantification was performed using the standard curve method.<sup>42</sup> The target quantity was divided by the endogenous (*Gapdh* or *Actb*) quantity to obtain a normalized target value.

**5.10. Measurement of IL-6, IL-1 $\beta$ , MCP-1, and NO Levels in the Culture Supernatant.** The levels of MCP-1, IL-6, and IL-1 $\beta$  in the culture supernatant were determined using a commercial ELISA kit. NO levels in the culture supernatant were determined using the Griess method.<sup>43</sup> After LPS stimulation for 24 h, the culture media were collected and mixed with an equal amount of Griess reagent (0.1% *N*-[1-naphthyl]ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid in distilled water), and then the absorbance at 550 nm was determined. To calculate NO levels, a standard curve was created using NaNO<sub>2</sub>. For the blank sample, the media containing carotenoids without cells were used.

**5.11. Statistics.** The results are represented as the mean  $\pm$  standard error of the mean (SEM). One-way ANOVA followed by the Tukey's honest significant difference (HSD) test were used to evaluate the statistical difference ( $P < 0.05$ ).

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c01164>.

Experimental details regarding synthesis of apo-15-astaxanthinal, (2E,4E)-6,6-dimethoxy-2-methylhexa-2,4-dienal, apo-14'-astaxanthinal, and apo-12'-astaxanthinal; pictures of differentiated 3T3-L1 adipocytes at day 10; and additional mRNA expression data of inflammatory cytokines in coculturing of RAW264.7 macrophages and 3T3-L1 adipocytes (PDF)

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N.T.: conceptualization, acquisition of data, funding acquisition, and writing of original draft. F.B.: conceptualization and editing of manuscript. Y.Y.: acquisition of data and editing of manuscript. T.M.: acquisition of data and editing of manuscript. K.M.: supervision and editing of manuscript. M.H.: conceptualization, funding acquisition, supervision, and editing of manuscript.

### Notes

The authors declare no competing financial interest.

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### ABBREVIATIONS USED

LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; IL, interleukin; Ptg2, prostaglandin-endoperoxide synthase 2; NO, nitric oxide; Nos2, nitric oxide synthase 2; Ccl2, C–C motif chemokine ligand 2; RAW-CM, RAW264.7 derived conditioned media

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