NOTE

Anti-oxidative, anti-tumor-promoting, and anti-carcinogenic activities of adonirubin and adonixanthin

Takashi Maoka¹*, Hiroyuki Yasaki², Aya Ohmori², Harukuni Tokuda³, Nobutaka Suzuki³, Ayako Osawa⁴, Kazutoshi Shindo⁴ and Takashi Ishibashi⁵

¹ Research Institute for Production Development (15 Shimogamo-morimoto-cho, Sakyou-ku, Kyoto 607-0805, JAPAN)
² Department of Analytical and Bioinorganic Chemistry, Division of Analytical and Physical Chemistry, Kyoto Pharmaceutical University (5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, JAPAN)
³ Department of Complementary and Alternative Medicine, Clinical R&D, Graduate School of Medical Science, Kanazawa University (13-1 Takara-machi, Kanazawa 920-8640, JAPAN)
⁴ Department of Food and Nutrition, Japan Women’s University (2-8-1, Mejirodai, Bunkyo-ku, Tokyo 112-8681, JAPAN)
⁵ Biotechnology Business Section, Life Science Department, JX Nippon Oil & Energy Corporation (17-35, Niizo Minami 3, Toda, Saitama 335-8502, JAPAN)

Abstract: Anti-oxidative, anti-tumor-promoting, and anti-carcinogenic activities of adonirubin and adonixanthin, which are biosynthetic intermediates from β-carotene to astaxanthin, were investigated. Both adonirubin and adonixanthin showed almost the same activities for inhibition of lipid peroxidation and quenching of singlet oxygen as those of astaxanthin. Furthermore, adonirubin and adonixanthin exhibited an inhibitory effect on Epstein-Barr virus early antigen activation in Raji cells and carcinogenesis of mouse skin tumors initiated by 7,12-dimethylbenz[a]anthracene and promoted by 12-O-tetradecanoylphorbol-13-acetate.

Key words: adonirubin, adonixanthin, anti-oxidative activity, anti-tumor-promoting activity, anti-carcinogenic activity

1 INTRODUCTION

Adonirubin (3-hydroxy-β,β-carotene-4,4′-dione) and adonixanthin (3,3′-dihydroxy-β,β-caroten-4-one) are biosynthetic intermediates from β-carotene to astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione)¹. They were first isolated from the petals of *Adonis annua*²,³. Adonirubin and adonixanthin are found in astaxanthin synthesize bacteria such as *Paracoccus* and *Agrobacterium*, green algae (*Haematococcus pluvialis*), yeast (*Xanthophyllomyces dendrorhous*), crustaceans, and salmonidae fish etc. as minor carotenoids along with astaxanthin¹.

Recently, astaxanthin studies have focused on several biological functions such as radical scavenging, singlet oxygen (¹O₂) quenching, anti-carcinogenesis, anti-diabetic, anti-obesity, anti-inflammatory, anti-melanogenesis, and immune enhancement activities etc.⁴⁻⁸. From the structural similarity, it was assumed that adonirubin and adonixanthin may have similar activity to astaxanthin. However, there were no suitable industrial sources of adonirubin and/or adonixanthin, so the biological activities of adonirubin and adonixanthin have not been studied¹⁻⁸.

*Paracoccus carotinifaciens* is an aerobic gram-negative, halophilic, astaxanthin-producing bacterium that produces adonirubin and adonixanthin as sub major carotenoids⁹. Some strains of *P. carotinifaciens* produce adonirubin and adonixanthin at about 30~20% of the total carotenoids. This bacterium is a good source of adonirubin and adonixanthin; therefore, we investigated the anti-oxidative, anti-tumor-promoting, and anti-carcinogenic activities of adonirubin and adonixanthin isolated from *P. carotinifaciens*. In the present paper, we describe the results of these studies.

2 EXPERIMENTAL

2.1 Reagents

Astaxanthin, adonirubin, adonixanthin, and canthaxanthin were prepared from *P. carotinifaciens*⁹ according to our routine methods¹⁰. They were characterized by ¹H,
13C-NMR and FAB MS data. β-Carotene was purchased from Wako Pure Chemical Co. (Osaka, Japan). Structures of carotenoids used in this study are indicated in Fig. 1.

12-O-tetradecanoylphorbol-13-acetate (TPA), 7,12-dimethylbenz[a]anthracene (DMBA), methyl linolate, and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMBN) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hematoporphyrin was purchased from Wako Pure Chemicals (Osaka, Japan). 2,2,6,6-Tetramethyl-4-piperidone-N-oxyl (4-oxo-TEMPO), and 2,2,6,6-tetramethyl-4-piperidone (TMPD) were purchased from Aldrich (Milwaukee, WI, USA).

2.2 Inhibition of lipid peroxidation

According to the methods described by Terao, carotenoids were dissolved in EtOH at a concentration of 2 mM (final concentration of 167 μM in the reaction mixture). The sample solution, 0.1 mL, was added to 1 mL of 0.1M methyl linolate solution[n-hexane/2-propanol (1/1, vol/vol)], and the solution was incubated at 37°C for 5 min. As a control, EtOH alone was used instead of the sample solution. The oxidation reaction was then performed by incubation with air at 37°C. At regular intervals, the oxidation reaction products, methyl linolate hydroperoxides, were quantified by high performance liquid chromatography (HPLC). HPLC was performed with a Hitachi L-6000 intelligent pump and an L-4250 UV-VIS detector. The following HPLC conditions were employed for the quantitative analysis of methyl linolate hydroperoxides: column Lichrosorb Si 100(5 μm particle size) (4.6 × 250 nm) (Merck); solvent system: 2-propanol/n-hexane (1/99, vol/vol); flow rate: 1 mL/min; and detection: 235 nm.

2.3 Quenching activity of 1O2 produced by a methylene blue-sensitized photooxidation system in EtOH solution

1O2 quenching activity was examined by measuring methylene blue the sensitized photooxidation of linoleic acid. Forty microliters of 0.05 mM methylene blue and 10 μL of 2.4 M linoleic acid with or without 40 μL carotenoid (final concentration 1–100 μM each dissolved in EtOH) were added to micro glass vials (5.0 mL). The vials were tightly closed with a screw cap and a septum, and the mixtures were illuminated at 7,000 lux at 22°C for 3 h in corrugated cardboard. Then, 50 μL of the reaction mixture was removed and diluted to 1.5 mL with EtOH, and absorbance at 235 nm was measured to estimate the formation of conjugates dienes. The value in the absence of carotenoid was determined and 1O2 repression activity was calculated relative to this reference value. Activity is indicated as the IC50 value representing the concentration at which 50% inhibition was observed.

2.4 Quenching activities of 1O2 produced by the hematoporphyrin-UVA system in an aqueous solution

ESR spectra were recorded at room temperature with a JEOL JES-FR30 spectrometer (Tokyo, Japan) using an aqueous quartz flat cell (Radical Research, Tokyo, Japan). TMPD was used as an 1O2-trapping agent. 1O2 was generated by ultraviolet light (UVA) irradiation to the hematoporphyrin (HP) solution. UVA was irradiated through a UVA filter at a dose of 800 mW/cm² using a Supercure-203S (San-Ei Electric MFG, Osaka, Japan), which was connected to the ESR cavity. The reaction mixtures contained 62.5 μM HP, 50 mM TMPD, and 625 μM astaxanthin, adonirubin, or adonixanthin dissolved in acetone at room temperature (22°C) in a total volume of 0.2 mL of 50 mM phosphate buffer (pH 7.5). ESR spectra were measured at 30 sec after the irradiation (24 J/cm²) of the reaction mixtures. The ESR signal intensity appearing at the lowest magnetic field in a triplet spectrum due to a TMPD-1O2 adduct (2,2,6,6-tetramethyl-4-piperidone-N-oxyl, 4-oxo-TEMPO) was expressed as the ratio to the third signal intensity from the low magnetic field according to the external standard, Mn(II) doped in MnO.

Fig. 1 Structure of carotenoids.
2.5 *In vitro* Epstein-Barr Virus (EVA) early antigen activation induction effect

EBV genome-carrying lymphoblastoid cells (Raji cells) derived from Burkitt’s lymphoma were cultivated in RPMI-1640 medium with 10% fetal bovine serum (FBS). The Raji cells were incubated for 48 h at 37°C in a medium containing n-butyric acid (4 nmol), TPA (32 pmol), and various amounts of test compounds. Smears were made from the cell suspension, and an indirect immunofluorescence technique. Details of the *in vitro* assay for EBV-EA induction have been reported previously.\(^{30}\)

2.6 *In vivo* two-stage carcinogenesis assay on mouse skin papillomas initiated by DMBA and promoted by TPA

The animals (specific pathogen-free female ICR-6-week old mice) were divided into four experimental groups, each with 10 mice. The backs of the mice were shaved with surgical clippers, and they were treated topically with 7,12-dimethylbenz[a]anthracene (DMBA) (100 μg, 390 nmol) in acetone (0.1 ml) as the initiator. One week after initiation, papilloma formation was promoted twice a week by the application of TPA (1 μg, 1.7 nmol) in acetone (0.1 ml) to the skin (control group) with TPA treatment alone. Experimental groups received topical application of astaxanthin (85 nmol), adonirubin (85 nmol), and adonixanthin (85 nmol) in acetone (0.1 ml), respectively, 1 h before the TPA treatment. The incidence and numbers of papillomas were monitored weekly for 20 weeks.\(^{30}\)

Experiments involving mice were conducted in accordance with Kanazawa University, Institute for Experimental Animals and use Committee Guidelines, under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

3 RESULTS AND DISCUSSION

3.1 Anti-oxidative activity of adonirubin and adonixanthin

Inhibitory activity of adonirubin and adonixanthin lipid peroxidation was monitored by measuring the accumulation of methyl linolate hydroperoxides during the incubation of methyl linolate with AMVN as a radical initiator with astaxanthin, canthaxanthin, and β-carotene as positive controls. Figure 2 shows the results of inhibitory activity for lipid peroxidation by carotenoids at the concentration of 2 mM (final concentration of 167 μM). Astaxanthin showed the strongest lipid peroxidation inhibition effect followed by adonirubin, adonixanthin, canthaxanthin, and β-carotene. Both adonirubin and adonixanthin showed slightly weaker activity than astaxanthin, which is a well known antioxidant for lipid peroxidation\(^{12,17}\) and also showed higher activity than canthaxanthin and β-carotene. Several investigators have reported an increased number of conjugated double bonds in carotenoids, and the presence of functional groups such as carbonyl and hydroxyl groups in carotenoids, enhance their antioxidant effects.\(^{10,12,13,17-19}\). Therefore, it was assumed that the strong inhibitory activity of lipid peroxidation shown by adonirubin and adonixanthin was due to the presence of a long conjugated polyene system (thirteen conjugated double bonds for adonirubin and twelve double bonds for adonixanthin) and the presence of a 3-hydroxy-4-keto-β-end group. Adonixanthin, having twelve double bonds, showed higher antioxidative activity for lipid peroxidation than canthaxanthin, having thirteen conjugated double bonds. Canthaxanthin had two carbonyl groups at 4 and 4’ position. On the other hand, adonixanthin had a 3-hydroxy-4-keto-β-end group and a 3-hydroxy-β-end group in molecule. As reported by Terao, the presence of a 3-hydroxy-4-keto-β-end group in carotenoid might enhance antioxidative activity for lipid peroxidation.\(^{12}\)

Carotenoids are excellent quenchers of O\(_2^*\) and prevent photooxidation. O\(_2^*\) quenching activities of adonirubin and adonixanthin were studied by a methylene blue-sensitized photooxidation system in EtOH solution and a hematoporphyrin-UVA system in an aqueous solution.

![Graph](image-url)
Figure 3 shows \( ^{1}O_2 \) quenching activity of astaxanthin, adonirubin, and adonixanthin in the methylene blue-sensitized photosensitization system in EtOH solution by carotenoids. Results of \( ^{1}O_2 \) quenching activities are expressed as the mean ± SD of three repeated experiments. Statistical analysis was performed by analysis of variance (ANOVA) followed by Student’s t-test. The same superscript in a row indicates no significant differences (p<0.05).
Biological activity of adonirubin and adonixanthin

Table 1 Relative rate of EBV-EA activation\(^a\) with respect to the positive control (100\%) in the presence of carotenoids.

<table>
<thead>
<tr>
<th>Concentration (mol ratio/TPA)(^b)</th>
<th>1000</th>
<th>500</th>
<th>100</th>
<th>10</th>
<th>IC(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compounds</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astaxanthin</td>
<td>8.5 ((60))^c</td>
<td>22.8</td>
<td>74.8</td>
<td>100</td>
<td>317</td>
</tr>
<tr>
<td>Adonirubin</td>
<td>12.9 ((60))</td>
<td>28.4</td>
<td>78.5</td>
<td>100</td>
<td>340</td>
</tr>
<tr>
<td>Adonixanthin</td>
<td>10.8 ((60))</td>
<td>26.9</td>
<td>76.2</td>
<td>100</td>
<td>329</td>
</tr>
</tbody>
</table>

\(^a\) Values represent the percentage relative to the positive control values (100\%).
\(^b\) TPA concentration was 20 ng (32 pmol)/mol.
\(^c\) Values in parentheses are percentage viability of Raji cells. There were significant differences \((P<0.01)\) in the inhibitory capacity of carotenoid treatment groups compared with the control group.

Fig. 5 Inhibitory effects of carotenoids on DMBA-induced mouse skin carcinogenesis. - - DMBA (390 nmol) + TPA (1.7 nmol) - - Adonirubin (85 nmol) + DMBA (390 nmol) + TPA (1.7 nmol) - - Adonixanthin (85 nmol) + DMBA (390 nmol) + TPA (1.7 nmol) - - Astaxanthin (85 nmol) + DMBA (390 nmol) + TPA (1.7 nmol) There were significant differences \((P<0.01)\) in the inhibitory capacity of carotenoid treatment groups compared with the control group.

Inhibitory effects on EBV-EA induction of Raji cells without significant cytotoxicity (more than 60\% viability of Raji cells) in this assay. Adonirubin and adonixanthin showed slightly weaker activity than astaxanthin, which has strong anti-tumor-promoting activity\(^{15}\).

Furthermore, the inhibitory effects of adonirubin and adonixanthin on two stage mouse skin carcinogenesis were investigated. The incidence of papilloma-bearing mice and the average number of papillomas per mouse in the astaxanthin, adonirubin, adonixanthin, and control groups are shown in Fig. 5. When astaxanthin (85 nmol), adonirubin (85 nmol) and adonixanthin (85 nmol) were applied before each TPA treatment, they markedly delayed the formation of papillomas and reduced the number of papillomas per mouse as shown in Fig. 5. Adonirubin and adonixanthin showed slightly weaker activity than astaxanthin, which is a well known anti-carcinogenic carotenoid\(^{20-23}\). These results indicated that adonirubin and adonixanthin had anti-tumor-promoting and anti-carcinogenic activities similar to astaxanthin.

4 CONCLUSION

In the present investigation, we revealed that adonirubin and adonixanthin had almost the same anti-oxidative, anti-tumor-promoting, and anti-carcinogenic activities as those of astaxanthin. Thus, adonirubin and adonixanthin may have the potential to reduce the risk of disease induced by oxidative stress and to prevent cancer similar to astaxanthin.

References


