The Antioxidant Effect of Palm Fruit Carotene on Skin Lipid Peroxidation in Guinea Pigs as Estimated by Chemiluminescence-HPLC Method

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Summary To study the antioxidant effect of palm fruit carotene on skin lipid peroxidation, the guinea pigs were orally fed ad libitum palm fruit carotene, β-carotene, or vehicle emulsions, in which carotene (0.05%, w/w) was suspended in drinking water. After treatment of carotene for 12 weeks, animals were exposed to ultraviolet ray (UV), and squalene monohydroperoxide (SqOOH)/squalene (Sq) ratios in the skin lipid were analyzed using the chemiluminescence-HPLC method. Carotene accumulation was found in the skin of guinea pigs that were orally administered palm fruit carotene or β-carotene. After UV irradiation, especially immediately after, the rise in the SqOOH/Sq ratio was effectively suppressed in both carotene-drinking groups in contrast with the control (carotene-untreated) group. An inverse correlation between the carotene content and the SqOOH/Sq ratio in the skin was also observed. The results suggested that palm fruit carotene intake prevents skin lipid peroxidation caused by UV irradiation.

Key Words palm fruit carotene, β-carotene, lipid peroxidation, squalene, hydroperoxide, ultraviolet ray, antioxidant, chemiluminescence, CL-HPLC

In a previous paper (1), we showed that palm fruit carotene intake was effective in preventing skin lipid peroxidation in hairless mice as estimated by thiobarbituric acid (TBA) assay. Although TBA assay is widely accepted as an indication of lipid peroxidation, it has the disadvantage for specific quantitative analysis of lipid peroxides in lipid class levels. Previously, Miyazawa et al. (2,3) established a chemiluminescence detection-high performance liquid chromatography (CL-HPLC) method for the quantitative assay of lipid hydroperoxides such as
phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) present in biological tissues at a detection limit of 10 pmol of hydroperoxide-O$_2$. The best advantage of this chromatographic method is its high specificity for the hydroperoxide group in each lipid molecule. During the photooxidation of the skin lipid, earlier reports have suggested that squalene (Sq) is one of the main sources of skin lipid peroxides (4–6). Kohno et al. (7, 8) were the first to show that the most susceptible lipid class in human skin lipid peroxidation is Sq and they quantitatively measured squalene monohydroperoxide (SqOOH) as a primary peroxidation product using the CL-HPLC method. In this study, by employing the CL-HPLC method, we quantitatively compared the antioxidant effect of palm fruit carotene and chemically synthesized β-carotene by oral intake on ultraviolet ray (UV)-dependent skin lipid peroxidation including the SqOOH formation in guinea pigs.

METHODS

**Chemicals.** Detailed descriptions of the reagents are given in our preceding paper (1). Squalene (Sq) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and purified by silica column chromatography. Squalene monohydroperoxide (SqOOH) was prepared by the photooxidation of squalene (8). Cytochrome c (from horse heart, type VI) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were purchased from Wako Pure Chemical Industries Co. (Osaka, Japan).

**Animals and dietary supplementation.** Four-week-old female albino Hartley guinea pigs (Kasyo Co., Tokyo, Japan; initial body weight, ca. 250 g) were used for the test. They were acclimatized in a room maintained at 25±1°C, 50±10% humidity with a 12 h light/dark cycle (lighting at 8:00–20:00) and fed solid RGRO feed (Nihon Nosan Kogyo Inc., Tokyo, Japan) and tap water. One week after arrival, palm fruit carotene (at a concentration of 0.05%), β-carotene (0.05%), or vehicle emulsion was dispersed in the drinking water and given ad libitum for 12 weeks. For preparation of the drinking water, each palm fruit carotene or β-carotene was suspended as an emulsion to a final concentration of 0.05% (w/w) in the water. The drinking water was prepared every 3 days. Carotene suspended in the drinking water was stable at least for 3 days. After 12 weeks of oral administration, the guinea pigs received UV treatment, and the total carotene and retinol content in the skin and SqOOH/Sq ratios in the skin lipid were analyzed.

**UV irradiation.** Eighteen guinea pigs had their backs shaved with an electric clipper 24 h before the UV irradiation. The condition of UV irradiation and the skin lipid sampling period were determined according to the method described in our previous paper (1) but with slight modification. The animals were fixed with 4 sections of 7 cm$^2$ area on their exposed backs, and received 5.0 J/cm$^2$ of UV from a DMR-100 (Eisai Co., Ltd., Tokyo, Japan). The UV-A emission was 3.8 J/cm$^2$ (4.6 mW/cm$^2$ for 833 s) and the UV-B was 1.2 J/cm$^2$ (1.4 mW/cm$^2$ for 833 s). Before 0.5
CAROTENE INTAKE AND SKIN LIPID PEROXIDATION

Scheme 1. Flow scheme for CL-HPLC assay of squalene monohydroperoxide of the skin lipid from guinea pigs.

h of irradiation and 0.5 (immediately), 1.5, and 3 h after irradiation, the skin lipid in each area was collected by wiping the skin surface with a small cotton pellet which was impregnated with a mixture of acetone and diethylether (1:1, v/v, containing 50 μM butylhydroxytoluene as an antioxidant). The cotton pellet was placed in 8 ml of diethylether, and the head space of the vessel was replaced with nitrogen gas and stored at −80°C until use. A flap of the dorsal skin in each animal, which was a shaded area that received UV irradiation, was surgically removed and stored at −80°C until use.

**Carotene and retinol assays.** Carotene and retinol in the skin were measured according to the method described in our previous paper (1). Data were given with M±SE for each of 6 animals and expressed as μg/g skin.

**CL-HPLC of the skin lipids.** SqOOH/Sq ratios in the skin surface lipid were measured according to the CL-HPLC method of Miyazawa et al. (2,3,7,8) (Scheme 1). The CL-HPLC was composed of a reverse phase isocratic system with a CAPCELL-pak C-18 column and methanol-ethanol = 5:1 (v/v) as the column.
Fig. 1. CL-HPLC chromatogram of photooxidized squalene, which contained 40.3 pmol of squalene monohydroperoxide (SqOOH) and 3.3 nmol of squalene (Sq). The CL-HPLC conditions were as follows: HPLC column, CAPCELL-pak C-18; mobile phase, methanol-ethanol = 5:1 (v/v), flow rate 1.1 ml/min; chemiluminescent reagent, 10 µg/ml cytochrome c and 1.0 µg/ml luminol dissolved in 50 mM borate buffer (pH 9.7), flow rate 1.0 ml/min. A, chemiluminescence detection; B, 210 nm detection.

mobile phase. The hydroperoxide-specific chemiluminescent reagent was a mixture of 10 µg/ml cytochrome c and 1.0 µg/ml luminol dissolved in an alkaline borate buffer (pH 9.7). SqOOH was detected using a CLD-110 chemiluminescence detector (Tohoku Electronic Industries Co., Sendai, Japan) and Sq detected at 210 nm with a UV detector. Values were expressed as the M±SE for each of 6 animals. Figure 1 shows a typical chromatogram of the photooxidated squalene, which contained 40.3 pmol of SqOOH (retention time of 5 min) and 3.3 nmol of Sq (11 min).

Statistical analysis. Any significant difference in the mean values between groups were determined using Tukey’s honestly significant differences test.

RESULTS

Body weight gain and carotene intake

There were no significant differences in the final body weight and in the amount of drinking water consumed among the groups (Table 1). The dose of palm fruit carotene and β-carotene, calibrated from the mean amount of drinking water consumed, is summarized in Table 1.

Carotene and retinol in the skin

Table 2 shows the total carotene and retinol content in the skin after 12 weeks of oral administration. In both carotene-dosed groups, significantly higher levels (p<0.05) of carotene accumulation in the skin were observed when compared with the control group. This indicates that oral intake of carotenes increases the carotene content in the skin of guinea pigs. The skin retinol content showed no difference among the three groups.

SqOOH/Sq ratio in the skin

Figure 2 shows typical chromatograms of the guinea pig skin lipids in CL-HPLC. In the control group, an SqOOH peak was not detected on the chemilumi-
CAROTENE INTAKE AND SKIN LIPID PEROXIDATION

Table 1. Body weight gain and carotene intake of guinea pigs during experimental period.

<table>
<thead>
<tr>
<th>Group</th>
<th>n²</th>
<th>Body Weight (g)¹</th>
<th>Drinking water consumed (ml/head/day)¹</th>
<th>β- or palm fruit carotene intake (mg/head/day)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>260±5</td>
<td>669±20</td>
<td>98±6</td>
</tr>
<tr>
<td>+ 0.05% β-carotene</td>
<td>6</td>
<td>255±7</td>
<td>617±37</td>
<td>101±7</td>
</tr>
<tr>
<td>+ 0.05% palm fruit carotene</td>
<td>6</td>
<td>250±5</td>
<td>654±10</td>
<td>100±4</td>
</tr>
</tbody>
</table>

Four-week-old female guinea pigs were used in this experiment. Palm fruit carotene (at the concentration of 0.05%), β-carotene (0.05%), or vehicle emulsion as control was suspended in drinking water and given per os for 12 weeks. ¹M±SE. ²represents number of guinea pigs.

Table 2. Carotene and retinol contents in the skin of guinea pig.

<table>
<thead>
<tr>
<th>Group</th>
<th>n¹</th>
<th>Carotene (µg/g skin)²</th>
<th>Retinol (µg/g skin)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0.06±0.01</td>
<td>0.23±0.02</td>
</tr>
<tr>
<td>+ 0.05% β-carotene</td>
<td>6</td>
<td>0.14±0.02³</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td>+ 0.05% palm fruit carotene</td>
<td>6</td>
<td>0.12±0.02³</td>
<td>0.29±0.02</td>
</tr>
</tbody>
</table>

Palm fruit carotene (at the concentration of 0.05%), β-carotene (0.05%), or vehicle emulsion as control was suspended in drinking water and given per os to guinea pigs for 12 weeks. A flap of dorsal skin was surgically removed and carotene and retinol content in the skin were measured with HPLC. The HPLC conditions were as follows: column, CAPCELL-pak C-18; mobile phase, acetonitrile-methanol-tetrahydrofuran=58:38:7 (v/v/v); flow rate, 1 ml/min. ¹represents number of guinea pigs. ²M±SE. ³p<0.05, as compared with control group.

nescence chromatogram before UV irradiation in spite of the presence of an Sq peak that appeared at 11 min retention time on the UV chromatogram (Fig. 2A and B). After UV irradiation, the SqOOH peak remarkably appeared at 5 min retention time on the chemiluminescence chromatogram (Fig. 2C and D). In the palm fruit carotene-drinking group, before UV irradiation, the chromatogram patterns were similar to those of the control group (Fig. 2E and F). After UV irradiation, the SqOOH peak was detected in the palm fruit carotene-drinking group (Fig. 2G and H) but was apparently smaller than that of the control group (Fig. 2C and D). Other peaks (10-40 min retention times) on the CL-HPLC chromatogram, which were recognized as other hydroperoxides such as triacylglycerol hydroperoxides formed on the skin, were also smaller for the palm fruit carotene-drinking group (Fig. 2G) as compared with those of the control group (Fig. 2C). The SqOOH/Sq ratio was tentatively calibrated from these chromatogram areas ascribed to SqOOH and Sq. Figure 3 shows the changes in the SqOOH/Sq ratio in the skin lipid in each
The conditions of carotene administration were the same as described in Table 2. The guinea pigs received 5.0 J/cm² of UV irradiation (3.8 J/cm² of UV-A and 1.2 J/cm² of UV-B). Before 0.5 h of irradiation (BI) and immediately (IA), 1.5 and 3 h after irradiation, the skin lipid was collected by wiping the skin with a small cotton pellet which was impregnated with a mixture of acetone and diethylether (1:1, v/v), and analyzed by CL-HPLC. The CL-HPLC conditions were the same as described in Fig. 1. A, control BI at CL detection; B, control BI at 210 nm detection; C, control IA at CL detection; D, control IA at 210 nm detection; E, palm fruit carotene BI at CL detection; F, palm fruit carotene BI at 210 nm detection; G, palm fruit carotene IA at CL detection; H, palm fruit carotene IA at 210 nm detection.

animal group. Before UV irradiation, the SqOOH/Sq ratios were not different among all experimental groups, but after UV irradiation, especially immediately after (IA in Fig. 3), the increase in the ratio was significantly suppressed in both carotene-drinking groups in contrast to the control group.

Correlation between carotene content and SqOOH/Sq ratio in the skin

The carotene content in the skin and the SqOOH/Sq ratio after UV irradiation are shown in Fig. 4. An inverse correlation was observed between the carotene
Fig. 3. Time-course of SqOOH/Sq ratios in the skin lipid from guinea pigs after UV irradiation. The UV irradiation was the same as described in Fig. 2. ■, control group; ▲, palm fruit carotene-drinking group; ○, β-carotene-drinking group; ↑, UV irradiation; BI, before irradiation; IA, immediately after irradiation. Each point represents the M±SE of 6 animals.

Fig. 4. Relationship between carotene content and SqOOH/Sq ratios of skin lipid from guinea pigs immediately after UV irradiation. The experimental conditions were the same as described in Table 2 (carotene administration) and in Fig. 2 (UV irradiation). ■, control group; ▲, palm fruit carotene-drinking group; ○, β-carotene-drinking group.

content and the SqOOH/Sq ratio and such relationship was most typically observed in the control group.

DISCUSSION

In the present experiment, we demonstrated that carotene administrated per os
in drinking water was accumulated in the skin of guinea pigs, and the UV-induced skin lipid peroxidation which was estimated from SqOOH/Sq ratio with CL-HPLC assay was suppressed by the oral intake of carotenoids, such as palm fruit carotene in guinea pigs. The carotene dosage in this study (50 mg/guinea pig/day in Table 1) was in greater excess than in the previous study (0.3 mg/mouse/day) (I). As already described in a previous paper about such excess carotene administration (I), the efficiency of absorption and accumulation are very different among animal species (9,10) and the serum carotene level in guinea pigs (11 mg/100 ml) barely matched the normal level in humans (30–40 mg/100 ml) (11). As shown in Table 2, in carotene-treated groups, the carotene content in the skin was higher than in the control group. For excess oral administration, the carotene accumulation was observed in the skin not only in hairless mice but also in guinea pigs as in humans. The skin carotene content of the control guinea pigs was higher than that of hairless mice reported in our previous paper (1). This may be due to differences in the solid feed. The \( \beta \)-carotene content in the solid feed for guinea pigs was 32 ppm while it was about 10-fold higher than that for mice. The guinea pigs took about 75 g of solid feed per day. Therefore, the control guinea pigs received about 2.4 mg/head/day carotene from the solid feed which was equivalent to almost 5% of the administered carotene from the drinking water in the carotene-treated groups. In our previous paper (1), we observed that the palm fruit carotene administration more efficiently increased the skin carotene content than that of \( \beta \)-carotene administration in the hairless mice. In guinea pigs, however, the carotene content and the accumulation ratio were not very different between the \( \beta \)-carotene and palm fruit carotene administration (Table 2).

Previous reports have suggested that Sq is the main origin of lipid peroxide during the photooxidation of human skin (4–6). Recently, Kohno et al. (7,8) were the first to quantitatively confirm the SqOOH formation in human skin due to sunlight exposure using the CL-HPLC method. In rodents, the lipid composition of the sebum was different and the Sq content was very low in contrast to that in human skin (12,13). As shown in Fig. 2A and C, the chemiluminescent SqOOH peak was most evident after UV irradiation in the control group. Several chemiluminescent peaks other than SqOOH were also observed in the skin lipid of guinea pigs (Fig. 2A, C, E, and G). These were presumably ascribed to hydroperoxides of triacylglycerols, wax esters, free fatty acids, and cholesterol esters, which also reflects the difference in skin lipid compositions. Although the components of chemiluminescent peaks other than SqOOH were also enhanced by UV irradiation, the highest appearance of SqOOH (Fig. 2A and C) suggested that Sq is one of the most susceptible lipid classes of skin lipids for UV-induced peroxidation. Ogura et al. (14,15) reported that the skin TBARS shows a significant increase at 3 h after UV irradiation in hairless mice. The present results clearly demonstrated that a significant elevation of skin SqOOH appeared immediately after UV irradiation in guinea pigs. This study also indicated that the CL-HPLC method is a useful tool for the quantification of hydroperoxides in lipid class levels formed in skin.

CAROTENE INTAKE AND SKIN LIPID PEROXIDATION

Many arguments have been made on the generation of reactive oxygen intermediates in the skin during UV radiation. Sam and Guan (16) reported that the initial reaction of Sq with singlet molecular oxygen occurs by allylic hydroperoxide formation at the terminal double bond. Kohno et al. (8) reported that human skin lipids after sunlight exposure have the same retention time of SqOOH detected by CL-HPLC like that of squalene monohydroperoxide which was prepared by the dye-sensitized photooxidation of Sq. The SqOOH detected in the present study was suggested to be formed by the singlet oxygen-involving photosensitization. As shown in Fig. 3, the SqOOH/Sq ratio was effectively suppressed by oral administration of carotene in guinea pigs. Carotenoids in skin may play a role as a singlet oxygen quencher and suppress the induction of SqOOH formation (17).

Mathews-Roth et al. (18, 19) reported the photoprotective ability of β-carotene during oral administration. In this study, we demonstrated the antioxidant function of oral palm fruit carotene. As shown in Fig. 2, oral administration of palm fruit carotene could suppress not only SqOOH formation but also other types of hydroperoxides. In the palm fruit carotene group, the SqOOH/Sq ratio, which was enhanced by UV irradiation, was maintained at a lower level compared with control group (Fig. 4). Natural carotenoids such that palm fruit carotene seemed to have a potent antioxidant activity. Mascio et al. (20) showed the higher singlet oxygen quenching ability of lycopene, γ-carotene and α-carotene as compared with that of β-carotene. Palm fruit carotene contains 30% α-carotene, 60% β-carotene, and 10% others (γ-carotene, lycopene, etc.). These constituents beside β-carotene may also contribute to the effectiveness of natural palm fruit carotene for preventing peroxidation of skin lipids.

REFERENCES


