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Cholesterol 7-Hydroperoxides in Rat Skin as a Marker for Lipid Peroxidation

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ABSTRACT. Concentrations of cholesterol 7α- and 7β-hydroperoxides (Ch 7-OOHs) in the skin of rats were determined by HPLC with a chemiluminescence detector. We demonstrated that (a) the concentrations of Ch 7-OOHs in rat skin were highly correlated with rat age ($r = 0.929$; N = 51, 1 to 55 weeks old), (b) the concentrations of Ch 7-OOHs in the skin of rats in an ambient light room were not significantly different from those found in rats kept in a dark room for 12 weeks, and (c) lipid peroxidation *in vitro* induced by ADP-Fe^{2+} caused an increase in the concentrations of Ch 7-OOHs in homogenates of rat skin. These results indicated that levels of Ch 7-OOHs in skin might be a good marker for aging of rats and might be independent of housing illumination, thus a good marker for endogenous lipid peroxidation. Furthermore, we observed that ultraviolet light B (UVB) irradiation markedly enhanced the concentrations of Ch 7-OOHs in the skin of rats *in vivo* depending on the duration of the irradiation, and the increases in Ch 7-OOHs were inhibited by radical scavengers, i.e. tocopherols. Therefore, it was suggested that the levels of Ch 7-OOHs in the skin could also be a good marker for UVB-dependent lipid peroxidation. BIOCHEM PHARMACOL **58**;9:1415–1423, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. aging; cholesterol hydroperoxide; HPLC; lipid peroxidation; rat skin; ultraviolet light

For the last decade, extensive investigations in many basic and applied science fields have been focused on reactive oxygen species, which have played important roles in a variety of diseases [1, 2] and in aging [3, 4]. One of the most basic mechanisms by which reactive oxygen species exert these biological effects is the induction of lipid peroxidation [5, 6]. Lipid peroxidation generates a variety of reactive free radicals, which modify and destroy tissue components such as lipids, proteins, and DNA [6, 7]. Skin is easily accessible and is exposed constantly and directly to air, sunlight, and air pollutants containing free radicals. Therefore, the role of reactive free radicals in aging [4, 8], carcinogenesis [9, 10], inflammation [11, 12], photosensitive syndrome [13], and UV-induced damage [14–16] of the skin has been investigated widely.

Cholesterol, an essential lipid constituent of biological membranes, is known to be oxidized under a variety of conditions to form oxidative products containing a hydroperoxy group. Whereas autoxidation of cholesterol under several conditions *in vitro* led to the formation of hydroperoxides, such as cholesterol 7α-, 7β-, 20α-, 24-, and 25-hydroperoxides, Ch 7α-OOH¶ and Ch 7β-OOH were the main products of cholesterol autoxidation *in vitro* at room temperature (Fig. 1) [17–19]. Furthermore, during lipid peroxidation in rat liver microsomes in the presence of ADP-Fe^{2+} and NADPH, Ch 7α-OOH and Ch 7β-OOH were the main hydroperoxy products of cholesterol [20]. Smith [21] and van Lier [22] have proposed a mechanism of the formation of Ch 7-OOHs from cholesterol *in vitro* that is initiated by lipid peroxidation of polyunsaturated fatty acids, which are more susceptible to oxidation than cholesterol.

In biological membranes, cholesterol and its 7-hydroperoxides exist in two different forms, namely the free form and the ester form containing saturated or unsaturated fatty acid. In a previous report [23], when tissue extract was treated with cholesterol esterase, two forms of Ch 7-OOHs could be determined separately by HPLC. We first demonstrated the close relationship between the concentrations of Ch 7-OOHs in rat skin and the age of rats by direct determination of the hydroperoxides using HPLC with a

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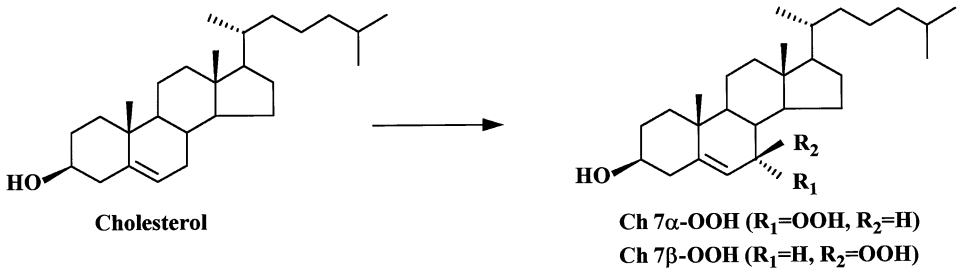
¶ *Abbreviations*: Ch 7α-OOH, cholesterol 7α-hydroperoxide (7α-hydroperoxycholest-5-en-3β-ol); Ch 5α-OOH, cholesterol 5α-hydroperoxide (5α-hydroperoxycholest-6-en-3β-ol); Ch 7β-OOH, cholesterol 7β-hydroperoxide (7β-hydroperoxycholest-5-en-3β-ol); Ch 7-OOHs, cholesterol 7-hydroperoxides; MANOVA, multivariate analysis of variance; MDA, malonaldehyde; and TBARS, thiobarbituric acid-reacting substances.



Cholesterol

Ch 7α-OOH (R_1=OOH, R_2=H)
Ch 7β-OOH (R_1=H, R_2=OOH)

FIG. 1. Cholesterol 7α- and 7β-hydroperoxides generated by free radical attack on cholesterol.

chemiluminescence detector [23]. In the present study, we quantitatively determined the free form and the free plus ester forms of Ch 7α-OOH and Ch 7β-OOH in rat skin under no-light, normal illumination (fluorescent light), and UVB exposure conditions. The results indicated that endogenous lipid peroxidation as well as UVB-induced lipid peroxidation enhances the formation of Ch 7-OOHs in rat skin, suggesting that Ch 7-OOHs are good markers for aging and photoaging.

MATERIALS AND METHODS

Chemicals

Ch 5α-OOH, [1α,2α(*n*)-^{3}H]Ch 5α-OOH, and β-sitosterol 5α-hydroperoxide (5α-hydroperoxystigmast-6-en-3β-ol) were synthesized by photooxidation of cholesterol, [1α,2α(*n*)-^{3}H]cholesterol, and β-sitosterol, respectively, in the presence of hematoporphyrin [24]. Ch 7α-OOH, [1α,2α(*n*)-^{3}H]Ch 7α-OOH, and β-sitosterol 7α-hydroperoxide (7α-hydroperoxystigmast-5-en-3β-ol) were obtained by isomerization of Ch 5α-OOH, [1α,2α(*n*)-^{3}H]Ch 5α-OOH, and β-sitosterol 5α-hydroperoxide, respectively, in chloroform [25]. Ch 7β-OOH, [1α,2α(*n*)-^{3}H]Ch 7β-OOH, and β-sitosterol 7β-hydroperoxide (7β-hydroperoxystigmast-5-en-3β-ol) were prepared from Ch 7α-OOH, [1α,2α(*n*)-^{3}H]Ch 7α-OOH, and β-sitosterol 7α-hydroperoxide, respectively, in ethyl acetate [26]. Their hydroxysterols were prepared from the respective hydroperoxides by reduction with sodium borohydride in methanol on ice. ADP, 5α-cholestane, cholesterol esterase (EC 3.1.1.13) from *Pseudomonas fluorescens*, microperoxidase (MP-11), β-sitosterol (minimum purity 95%), α-tocopherol, and γ-tocopherol were purchased from the Sigma Chemical Co., and [1α,2α(*n*)-^{3}H]cholesterol was obtained from Amersham International plc. l-Ascorbic acid (sodium salt), *N,O*-bis(trimethylsilyl)trifluoroacetamide, diethylenetriaminepentaacetic acid, 2,5-dimethylfuran, butylated hydroxytoluene, sodium borohydride, thiobarbituric acid, and 1,1,3,3-tetraethoxypropane were products of Wako Pure Chemical Industries. Other chemicals used were of reagent grade.

Animals

Male Sprague–Dawley rats were obtained from Charles River Japan Inc. Fifty-one rats (N = 3/age) 1- to 55-weeks-old were used to investigate the relationship between the concentrations of Ch 7-OOHs in rat skin and the age of rats or the cholesterol levels in skin. One-week-old rats (N = 5) were purchased and housed continuously for 12 weeks in an animal room with a normal light (fluorescent lamp: 12-hr light/dark cycle) or in a dark animal room to investigate the effects of housing illumination on concentrations of Ch 7-OOHs in rat skin. Nine-week-old rats were used for an *in vitro* study (N = 3) using ADP-Fe^{2+} and an *in vivo* study (N = 4) using UVB irradiation, in which rats were irradiated with UVB (20 mJ/cm^2/min) by using a portable UVB-irradiation lamp (280–320 nm, 15 W x 2; ATTO HP-30M, ATTO Co.) from above the cage. To estimate the effects of tocopherols on increases in Ch 7-OOHs induced by UVB irradiation, tocopherols were mixed into Vaseline™ (25 and 50%, w/w) and were applied to the skin of the backs of the animals (5 and 10 mg tocopherol/cm^2 skin, respectively). The animals were given tap water and food (CRF-1, Charles River Japan Inc.) *ad lib.*

Preparation of Rat Skin Homogenates for Analysis of Cholesterol and Ch 7-OOHs

Dorsal skin of rats was shaved and excised under general anesthesia, and lipid tissues were removed from the skin. The skin (1 g) used for analysis contained both epidermis and dermis and was homogenized for 10 min on ice with a Physcotron™ (Nichion, Tokyo) in 5 mL of saline containing 0.1% diethylenetriaminepentaacetic acid and 30 mL of chloroform:methanol (2:1, v/v) containing 0.1% butylated hydroxytoluene, 0.015% 2,5-dimethylfuran, and 5 nmol of β-sitosterol 7β-hydroperoxide as an internal standard.

Analysis of Ch 7α-OOH and Ch 7β-OOH in Rat Skin

Concentrations of Ch 7α-OOH and Ch 7β-OOH in rat skin were determined using HPLC with a chemiluminescence detector as reported previously [23, 27].

Analysis of Radiolabeled Ch 7α-OOH and Ch 7β-OOH Added to Rat Skin Homogenates

Quantitative determination of the radiolabeled Ch 7α-OOH and Ch 7β-OOH was conducted by the analysis method for Ch 7α-OOH and Ch 7β-OOH in rat skin, except for the final HPLC with a chemiluminescence

detector, in which the fractions of each radiolabeled cholesterol hydroperoxide were collected without a chemiluminescence detector for quantitative measurement of the radioactivity with a liquid scintillation counter (LSC-3500, Aloka).

In Vitro *Incubation System of Rat Skin Homogenates*

Dorsal skin homogenates [1 g skin equivalent; 4% homogenates (w/v) in 0.1 M sodium-potassium phosphate-buffered saline, pH 7.4] were prepared for incubation at 37° with ADP-Fe^{2+} (ADP: 5 mM, Fe^{2+}: 0.1 mM) and sodium ascorbate (1 mM) for 30, 60, and 120 min (N = 3/time point). In a stability study of radiolabeled Ch 7-OOHs, [1α,2α(*n*)-^{3}H]Ch 7α-OOH and [1α,2α(*n*)-^{3}H]Ch 7β-OOH (250 nmol each) were added to the same homogenates (N = 3) and then incubated for 10 and 30 min.

Analysis of Cholesterol in Rat Skin

Concentrations of cholesterol in homogenates of rat skin were determined by GC before and after hydrolysis with cholesterol esterase. Cholesterol and 5α-cholestane, used as an internal standard, were derivatized to trimethylsilyl ethers with *N,O*-bis(trimethylsilyl)trifluoroacetamide. The analytical instrumentation and conditions used were as follows: GC system 5890A (Hewlett Packard Co.); column DB-1, 0.25 mm × 15 m (J&W Scientific); oven temperature, 220°; injection port temperature, 250°; and flow rate of carrier gas, 0.9 mL helium/min. The retention times of cholesterol and 5α-cholestane were 14.9 and 6.0 min, respectively.

Assay of TBARS in Skin Homogenates of Rats

TBARS in skin homogenates were measured spectrophotometrically using 1,1,3,3-tetraethoxypropane as the MDA standard [28]. The values were expressed as MDA equivalents per gram of skin.

Statistical Analysis

Statistical analysis was performed using the Statistical Analysis System (SAS). Statistical significance was defined as $P < 0.05$.

RESULTS

Concentrations of Ch 7-OOHs in the Skin of 1- to 55-Week-Old Rats and Their Relationship with the Age of the Rats

Ch 7-OOHs, existing as free and ester forms in the skin of rats 1 to 55 weeks old, were determined quantitatively by HPLC with a chemiluminescence detector. In our assay method, the lower limits of quantitation for Ch 7α-OOH and Ch 7β-OOH were 0.1 and 0.5 nmol/g skin, respectively. The difference of the lower limits of quantitation between the epimers of Ch 7-OOHs was due mainly to the difference in sensitivity of each hydroperoxide itself to chemiluminescence detection; namely, the peak area ratio of chemiluminescence intensity Ch 7α-OOH:Ch 7β-OOH was approximately 5:1. A good correlation was observed between the concentrations of free plus ester forms of Ch 7-OOHs in rat skin and the age of the rats (Fig. 2A: r = 0.929; N = 51, 1 to 55 weeks old). On the other hand, the concentrations of Ch 7-OOHs in skin did not correlate well with the concentrations of cholesterol in free and ester forms (Fig. 2B: r = 0.626, N = 51, 1 to 55 weeks old). Concentrations of cholesterol in free form (6.61 ± 2.02 μmol/g skin) and in ester form (6.18 ± 1.95 μmol) were independent of rat age from 1 to 55 weeks (N = 51). The concentrations of ester forms of Ch 7-OOHs were approximately 8-fold higher than those of the free form in the skin of rats of all ages. The predominant epimer of Ch 7-OOHs in the skin was Ch 7β-OOH in rats of all ages; the ratios of Ch 7β-OOH to Ch 7α-OOH were 2.0 to 3.5 for the free

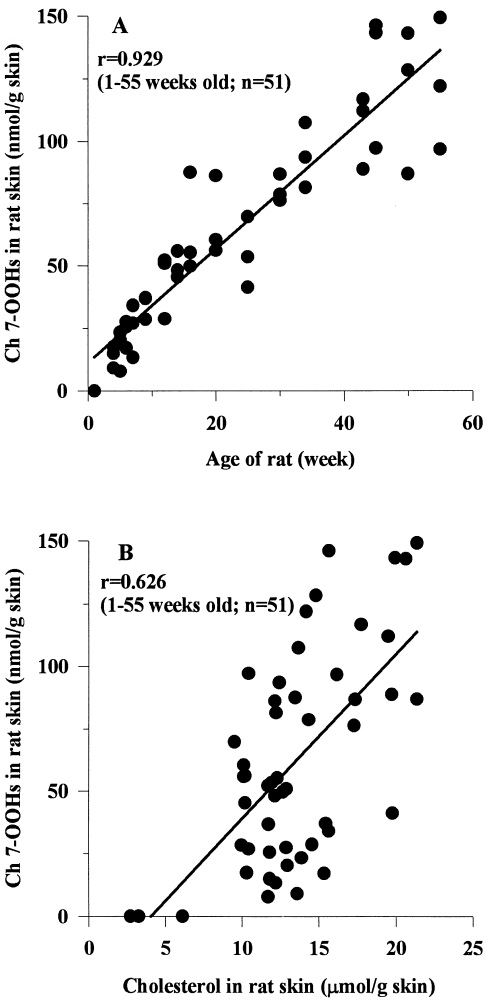


FIG. 2. Correlation between concentrations of Ch 7-OOHs in skin and age of rats (A) and cholesterol in skin (B). Concentrations of Ch 7-OOHs and cholesterol in free plus ester forms were determined in dorsal skin of rats 1 to 55 weeks old (N = 51).

TABLE 1. Recovery of [1α,2α(*n*)-^{3}H]Ch 7α-OOH and [1α,2α(*n*)-^{3}H]Ch 7β-OOH from skin homogenates of 7- and 20-week-old rats

Age of rat (weeks old)	Incubation time (min)	Recovery of [1α,2α(*n*)-^{3}H]Ch 7-OOHs* (%)	
		Ch 7α-OOH	Ch 7β-OOH
7	10	88.1 ± 3.9	86.9 ± 2.4
	30	85.5 ± 6.2	84.5 ± 3.8
20	10	86.7 ± 5.6	85.8 ± 4.8
	30	87.4 ± 5.5	86.9 ± 7.3

Values are means ± SD (n = 3). Recoveries of Ch 7-OOHs are corrected for absolute recovery in the assay method for free form i.e. 89.4 ± 3.4 and 86.6 ± 4.1% for [1α,2α(*n*)-^{3}H]Ch 7 OOh and [1α,2α(*n*)-^{3}H]Ch 7β-OOh, respectively.

* Recoveries of Ch 7-OOhs from rat skin homogenates were not significantly different between 7- and 20-week-old rats and between 10- and 30-min incubation (MANOVA: $P > 0.05$).

form and 5.1 to 9.8 for the free plus ester forms. These ratios were independent of the age from 1 to 55 weeks (N = 51). No significant differences in the concentrations of the hydroperoxides were observed in different parts of the dorsal skin (data not shown).

Recoveries of Radiolabeled Ch 7-OOHs from Rat Skin Homogenates

[1α,2α(*n*)-^{3}H]Ch 7α-OOH and [1α,2α(*n*)-^{3}H]Ch 7β-OOH (250 nmol each) were incubated with skin homogenates of 7- and 20-week-old rats at 37° for 10 and 30 min (Table 1). Absolute recoveries of radiolabeled Ch 7α-OOH and Ch 7β-OOH (250 nmol each) from skin in the assay method for the free form were 89.4 ± 3.4% (mean ± SD, N = 3) and 86.6 ± 4.1%, respectively, whereas those for free plus ester forms were 82.7 ± 4.4 and 80.8 ± 6.1%, respectively. Recoveries of radiolabeled Ch 7-OOHs corrected for the absolute recoveries in the assay method for the free form were 84.5 to 88.1% in skin homogenates of 7-week-old rats and 85.8 to 87.4% in those of 20-week-old rats. There were no significant differences in the recoveries between 7- and 20-week-old rat skin and between 10- and 30-min incubation (MANOVA, $P > 0.05$).

Effects of Housing Illumination on Concentrations of Ch 7-OOHs in Rat Skin

The effect of housing illumination on concentrations of Ch 7-OOHs in the skin was investigated in two groups of rats (N = 5/group) housed in a room with normal lighting or in a dark room continuously for 12 weeks. Concentrations of free plus ester forms of Ch 7α-OOH plus Ch 7β-OOH in the dorsal skin of rats were 43.6 ± 8.4 nmol/g skin in the ambient light room and 39.6 ± 11.8 nmol/g skin in the dark room (Fig. 3). The two groups of rats did not show any significant difference in the concentrations of Ch 7-OOHs in the dorsal skin (MANOVA, $P > 0.05$). In addition, no significant differences were observed in the Ch 7β-OOH/Ch 7α-OOH ratio (3.5 and 3.9 for the ambient light room and the dark room, respectively) or the ester/free form ratio (3.4 and 4.8 for light and dark rooms, respectively) between the two groups of rats (MANOVA, $P > 0.05$).

Increases in Concentrations of Ch 7-OOHs in Rat Skin Homogenates with ADP-Fe^{2+}

The time course for the formation of Ch 7-OOHs during lipid peroxidation induced by ADP-Fe^{2+} and sodium ascorbate was investigated in dorsal skin homogenates prepared from 9-week-old rats (Fig. 4). The concentration of the free plus ester forms of Ch 7-OOHs in skin homogenates linearly increased from 41.0 nmol/g skin at 0 min to 111 nmol/g skin at 120 min. During the first 60 min of incubation, however, the concentration of Ch 7-OOHs in the free form did not increase, but the ester form increased linearly. Thereafter, the concentrations of Ch 7-OOHs in the ester form approached a plateau phase, while the free form concentrations increased rapidly. Therefore, the ratio of ester form to free form in Ch 7-OOHs gradually increased from 8.9 at 0 min to 12.9 at 30 min and 15.7 at 60

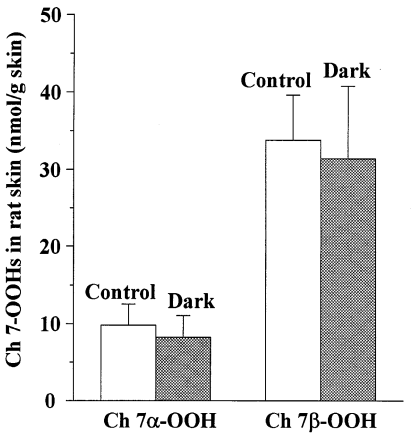


FIG. 3. Effects of housing illumination on concentrations of free plus ester forms of Ch 7-OOHs in rat skin. Rats in each group (N = 5) were purchased at the age of 1 week and housed in a room with normal light (fluorescent lamp: 12-hr light/dark cycle) or in a dark room all day for 12 weeks. Values are means ± SD (N = 5). There was no significant difference between the two groups (MANOVA: $P > 0.05$).

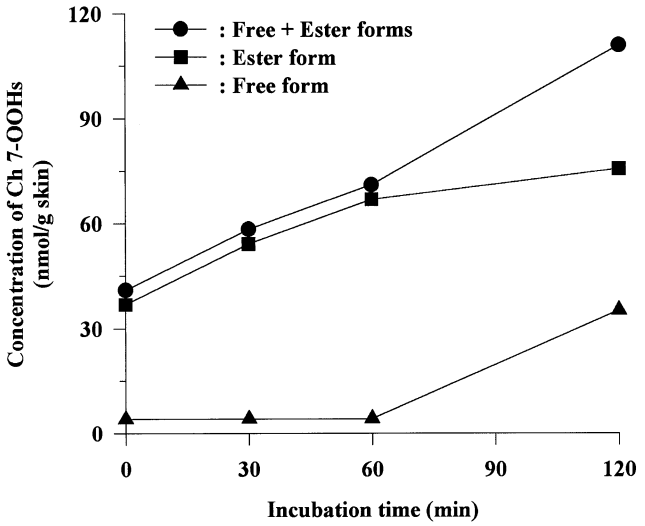


FIG. 4. Increases in Ch 7-OOHs in rat skin homogenates after the addition of ADP-Fe^{2+}. Skin homogenates [1 g skin equivalent; 4% homogenates (w/v) in 0.1 M sodium-potassium phosphate buffered saline, pH 7.4] were prepared from 9-week-old rats (N = 3) and were incubated at 37° with ADP-Fe^{2+} (ADP: 5 mM; Fe^{2+}: 0.1 mM) and sodium ascorbate (1 mM) for 30, 60, and 120 min. Values are means (N = 3).

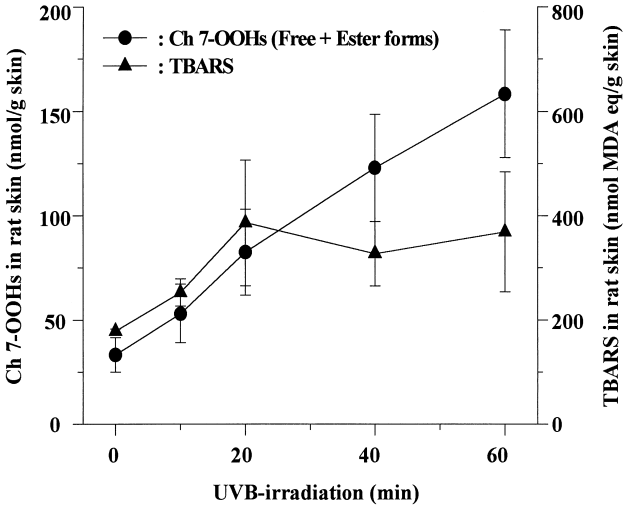


FIG. 5. Increases in Ch 7-OOHs and TBARS in rat skin during UVB irradiation. Rats at 9 weeks old were irradiated with UVB (20 mJ/cm^2/min) by using a portable UVB-irradiation lamp (280–320 nm) from above the cage for 10, 20, 40, and 60 min. Values are means ± SD (N = 4).

min, and then decreased to 2.1 at 120 min. The ratios of Ch 7β-OOH/Ch 7α-OOH were not changed time-dependently during incubation (2.0 to 2.1 in free form and 4.8 to 8.6 in free plus ester forms).

Increases in Concentrations of Ch 7-OOHs in Rat Skin Induced by UVB Irradiation

Irradiation of 9-week-old rats with a UVB lamp (280–320 nm) increased the levels of Ch 7-OOHs in the dorsal skin in a time-dependent manner (Table 2 and Fig. 5). After UVB irradiation for 60 min, the concentration of free plus ester forms of Ch 7-OOHs reached the maximum level, 158 ± 31 nmol/g skin, which was higher than that in the skin of 55-week-old rats. The levels of free plus ester forms of Ch 7-OOHs increased linearly from 0 to 60 min (Fig. 5). On the other hand, the ester form of Ch 7-OOHs increased rapidly for 40 min, and then slowed down. The free form of Ch 7-OOHs remained constant from 0 to 10 min and increased thereafter. The ratio of the ester form to free form of Ch 7-OOHs decreased from 6.9 at 0 min to 1.5 at 60 min (Table 2). UVB irradiation did not influence the ratios of Ch 7β-OOH/Ch 7α-OOH in free form (1.8 to 2.4) and free plus ester forms (4.5 to 6.1). On the other hand, TBARS in rat skins increased in a nonlinear fashion from 179 nmol MDA equivalents/g skin at 0 min to 327–386 nmol MDA equivalents/g skin at 20–60 min in response to UVB irradiation (Fig. 5).

Effects of Tocopherols on the Formation of Ch 7-OOHs in Rat Skin Induced by UVB Irradiation

The effects of α- and γ-tocopherols on the increase in the Ch 7-OOHs in dorsal skin of rats induced by UVB irradiation for 60 min were investigated (Fig. 6). Control values were obtained from rats to which Vaseline™ containing no tocopherols had been applied. The formation of the free plus ester forms of Ch 7-OOHs by UVB was reduced to 55 and 35% of the control by application of Vaseline™ containing α-tocopherol at 5 and 10 mg/cm^2, respectively. On the other hand, the formation of Ch 7-OOHs was reduced to 73 and 60% of the control by

TABLE 2. Increases in free and ester forms of Ch 7-OOHs in rat skin by UVB irradiation

UV-irradiation time (min)	Concentration of Ch 7-OOHs (nmol/g skin)		Ratio
	Free form	Ester form	Ester/Free
0	4.8 ± 2.4	28.5 ± 6.2	6.9 ± 2.5
10	7.9 ± 3.6	45.2 ± 10.9	6.3 ± 1.9
20	18.9 ± 3.8	63.5 ± 18.2	3.4 ± 0.9
40	36.9 ± 18.1	85.9 ± 20.5	2.7 ± 1.1
60	66.4 ± 15.4	92.2 ± 26.7	1.5 ± 0.7

Values are means ± SD (N = 4). Rats at 9 weeks old were irradiated with UVB (20 mJ/cm^2/min) by using a portable UVB-irradiation lamp (280–320 nm) from above the cage for 60 min.

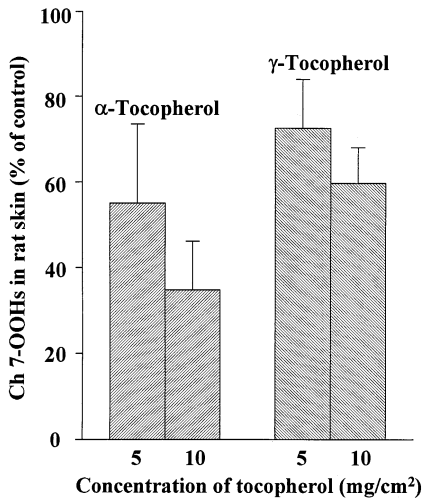


FIG. 6. Effects of α- and γ-tocopherols on the increase in Ch 7-OOHs in rat skin by UVB irradiation. Tocopherols were mixed into Vaseline™ (25 and 50%, w/w) and were applied to the skin of the backs of animals (5 and 10 mg tocopherol/cm^2 skin, respectively). UVB irradiation for 60 min was done under the same conditions as in Fig. 5. Values are means ± SD (N = 4). Absolute values were 154 ± 24 nmol/g skin in the control group, 84.4 ± 28.4 and 53.3 ± 17.4 nmol/g skin in the α-tocopherol group at 5 and 10 mg/cm^2, respectively, and 111 ± 18 and 91.7 ± 12.8 nmol/g skin in the γ-tocopherol group at 5 and 10 mg/cm^2, respectively. The three groups showed significant differences in the concentrations of Ch 7-OOHs in the skin of rats (MANOVA: $P < 0.05$).

γ-tocopherol at 5 and 10 mg/cm^2, respectively. Therefore, α-tocopherol was more efficient than γ-tocopherol in the inhibition of the formation of Ch 7-OOHs by UVB irradiation.

DISCUSSION

We have developed an assay method for the determination of Ch 7α-OOH and Ch 7β-OOH by HPLC with a chemiluminescence detector using β-sitosterol 7β-hydroperoxide as an internal standard [23]. We determined the free form and the free plus ester forms of each epimer of Ch 7-OOHs identified by (a) retention times in HPLC with normal phase, reverse phase, and chiral phase columns, and (b) MS for the hydroperoxides and GC-MS for their corresponding hydroxyl derivatives trimethylsilylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide following reduction with sodium borohydride [23]. In the present study, we demonstrated that the concentrations of Ch 7-OOHs in rat skin were highly correlated with rat age (r = 0.929; Fig. 2A). Our previous study indicated that the sum of concentrations of free and ester forms of Ch 7-OOHs correlated well with rat age (r = 0.874; N = 30, 1 to 45 weeks old) [23]. This result was confirmed by the present study, and a better correlation was observed because of the increase in the number of animals (r = 0.929; N = 51, 1 to 55 weeks old).

The concentrations of Ch 7-OOHs in rat skin increased linearly with age (Fig. 2A); the levels of Ch 7-OOHs in the skin of rats housed in an ambient light room and in a dark room were almost identical (Fig. 3). The latter indicated that the levels of Ch 7-OOHs in rat skin were not affected by ambient illumination. The *in vitro* study using the skin homogenates indicated that lipid peroxidation by ADP-Fe^{2+} caused a marked increase in the concentrations of Ch 7-OOHs (Fig. 4). Therefore, a close relationship between the concentrations of Ch 7-OOHs in rat skin and the age of rats suggests that Ch 7-OOHs in rat skin may be a good marker for aging of rats housed under normal conditions, namely a good marker for endogenous lipid peroxidation. On the other hand, the *in vivo* study indicated that UVB irradiation enhanced the concentrations of Ch 7-OOHs in rat skin (Table 2 and Fig. 5), and its increase was diminished by inhibitors of lipid peroxidation, i.e. tocopherols (Fig. 6). α-Tocopherol was more efficient than γ-tocopherol in inhibition of the formation of Ch 7-OOHs. Tocopherols may act partially by absorbing UVB (maximum absorbance spectrum at 295 nm) and generating tocopheroxyl radicals [29, 30], whereas it has been reported that tocopherols prevent oxidative damage primarily by scavenging peroxyl radicals and preventing propagation of lipid peroxidation through formation of the resonance-stabilized tocopheroxyl radicals [31, 32]. The scavenging potential of tocopherol derivatives decreased in the order of α-tocopherol > β-tocopherol > γ-tocopherol [33, 34]. The low molecular extinction coefficients of α-, β-, and γ-tocopherols are 71.0, 86.4, and 92.8, respectively. These findings suggest that tocopherols probably act partially by scavenging free radicals to inhibit increases in Ch 7-OOHs. Many reports [14–16] have indicated that UVB irradiation causes increases in lipid peroxides, measured as TBARS, in tissues of a living body. Furthermore, it has been suggested that TBARS formed in skin are leaked to the blood and then metabolized in the liver, since TBARS increase nonlinearly during UVB irradiation [16]. In the present study, it was observed that UVB irradiation enhanced the linear increase in the levels of free plus ester forms of Ch 7-OOHs and the nonlinear increase in TBARS in rat skins, depending on the duration of UVB irradiation (Fig. 5). Therefore, levels of Ch 7-OOHs in skin may be a better marker than TBARS for UVB-dependent lipid peroxidation.

The concentrations of Ch 7-OOHs in the ester form were higher than those in the free form in rats of all ages. However, the concentrations of cholesterol in free form and ester form were almost equal (6.61 ± 2.02 and 6.18 ± 1.95 μmol/g skin, respectively) in the skin of rats of all ages (N = 51, 1 to 55 weeks old). Furthermore, the *in vitro* and *in vivo* studies showed that the ester forms of Ch 7-OOHs increased rapidly compared with the free form from the early phase of lipid peroxidation caused by ADP-Fe^{2+} (Fig. 4) or UVB irradiation (Table 2 and Fig. 5). The *in vitro* stability study using radiolabeled Ch 7-OOHs indicated that Ch 7-OOHs were stable in skin homogenates of 7- and 20-week-old rats during a 30-min incubation at 37°. Al-

though we have no evidence for *in vivo* stability of Ch 7-OOHs in free and ester forms, it would appear that the *in vivo* stability of Ch 7-OOHs is not different between the free form and the ester form. If this is true, the cholesteryl ester is likely to be more susceptible to hydroperoxidation than the free form in a living animal. The unsaturated fatty acid moieties in the cholesteryl ester may contribute to its susceptibility to hydroperoxidation. The allylic hydrogen of the unsaturated fatty acid could be a labile site for free radical reaction with reactive oxygen species. The resulting free radicals could react in turn with the C7 hydrogen of the cholesterol moiety of the cholesteryl ester to form Ch 7-OOHs in the ester form. Furthermore, the cholesteryl esters are more lipophilic than the free form, and oxygen is more soluble in lipophilic sites than hydrophilic sites. Occurrences of cholesteryl ester hydroperoxides in biological specimens also have been reported [35–37]. We propose that Ch 7-OOHs may be formed *in vivo* by the following mechanism (Fig. 7): (a) a carbon radical at the C7 position of cholesterol is generated by the homolytic fission of the C—H bond by attack of radical species formed during lipid peroxidation in polyunsaturated fatty acids, (b) the resultant radical at the C7 position may react with molecular oxygen to form cholesterol 7α- and 7β-peroxyradicals, and (c) the addition of a hydrogen radical to the peroxyradicals may form Ch 7α-OOH and Ch 7β-OOH. Smith [21] and van Lier [22] also have proposed the above mechanism in the *in vitro* formation of Ch 7-OOHs. As compared with Ch 7-OOHs, Ch 5α-OOH is formed by a different mechanism, namely, photosensitized oxygenation of cholesterol, in which singlet oxygen is known to be an exclusive active reactant for the regiospecific lipoxygenation [24]. We have developed an assay method for Ch 5α-OOH using HPLC with a chemiluminescence detector [27, 38]. Concentrations of Ch 5α-OOH in the skin of rats under all conditions used in the present study were below the lower limit of quantitation (<0.20 nmol/g skin). Therefore, Ch 7-OOHs seem to be a main product formed from cholesterol by endogenous and UVB-dependent lipid peroxidation.

In many tissues, there are enzyme systems for metabolism of hydroperoxides and reactive oxygen species, such as phospholipid hydroperoxide glutathione peroxidase [39], glutathione peroxidase [40, 41], catalase [42, 43], and superoxide dismutase [44, 45]. A possibility that such enzyme activities in the skin decrease with aging [40, 46, 47] or in response to UVB irradiation [43, 48, 49] could not be dismissed. The *in vitro* stability study using radiolabeled Ch 7α-OOH and Ch 7β-OOH showed that recoveries of each radiolabeled hydroperoxide from the skin homogenates of 7- and 20-week-old rats were excellent (85–88%) after incubation at 37° for 30 min (Table 1). The addition of glutathione to the skin homogenates did not affect the recoveries of Ch 7-OOHs (data not shown). We have reported that in rats of any age, the liver had no detectable level of Ch 7-OOHs, and that Ch 7-OOHs were glutathione-dependently reduced to the corresponding alcohols by selenium-containing glutathione peroxidases and only by the α-class glutathione *S*-transferases Ya-Ya and Ya-Yc among all isoforms of glutathione *S*-transferases occurring in the liver [50]. On the other hand, rat skin had a very low level of glutathione peroxidases compared with the liver and lacked the subunit Ya-bearing glutathione *S*-transferases [50]. These findings strongly suggest that the reasons for the presence of Ch 7-OOHs in rat skin and the increase in the levels of Ch 7-OOHs with increasing age of rats or by UVB irradiation to rats may be attributable in large part to the absence of the α-class glutathione *S*-transferases bearing subunit Ya and to the presence of only a very low concentration of selenium-containing glutathione peroxidases in dermal tissues.

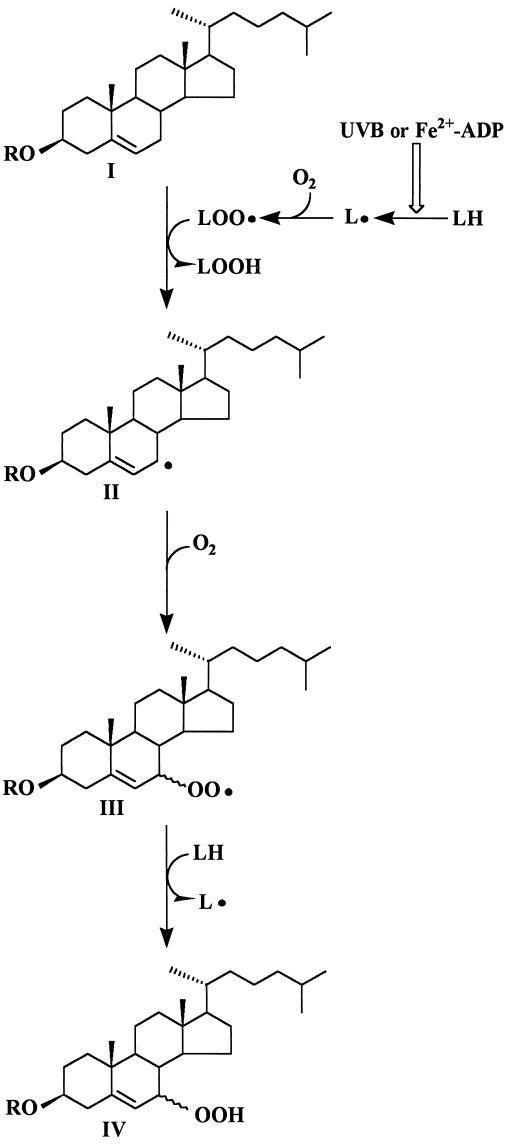


FIG. 7. Proposed mechanism for lipid peroxidation-mediated formation of 7α- and 7β-hydroperoxides of cholesterol and their fatty acid esters. LH represents polyunsaturated fatty acid residue of lipids and generates bi-allylic carbon radicals by UVB or ADP-Fe^{2+}. I: Cholesterol (Ch: R = H) or cholesteryl ester (R = fatty acyl); II: Ch 7-carbon radicals; III: Ch 7-peroxide radicals; and IV: Ch 7α- and 7β-hydroperoxides.

Not only cholesterol hydroperoxides but also hydroperoxides of other lipid components, e.g. phospholipids, triglycerides, and other steroids, could exist in the skin of rats, and these hydroperoxides may be increased by aging and UVB irradiation. It has been strongly suggested that skin cancer in humans is increased by UVB exposure [15, 51]. Our data indicated that concentrations of free plus ester forms of Ch 7-OOHs in skin lipids, collected with acetone-wet cotton swabs from human arms, were increased significantly by sunlight of early summer [52]. It is of primary importance to determine if the hydroperoxide levels correlate with skin cancer.

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