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Occurrence of singlet oxygen oxygenation of oleic acid and linoleic acid in the skin of live mice

YUKO MINAMI, KANAKO YOKOYAMA, NORIKO BANDO, YOSHICHIKA KAWAI, & JUNJI TERAO

Department of Food Science, Graduate School of Nutrition and Bioscience, The University of Tokushima, Tokushima 770-8503, Japan

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Abstract

To assess the contribution of singlet molecular oxygen [O_2 ($^1\Delta_g$)] to lipid peroxidation *in vivo*, this study combined gas chromatography-mass spectrometry with thin layer chromatography to analyse peroxidized lipids in the skin of hairless mice. Hydroxyoctadecenoate isomers and unconjugated hydroxyoctadecadienoate isomers derived from peroxidized oleic acid and linoleic acid, respectively, which are specific to O_2 ($^1\Delta_g$)-dependent oxygenation, were detected in the skin of live mice under ordinary feeding conditions. Short-term ultraviolet A (UVA)-irradiation of the skin *in vivo* elevated levels of the unconjugated hydroxyoctadecadienoate isomers significantly, whereas the irradiation of skin homogenate *in vitro* increased levels of all isomers derived from both O_2 ($^1\Delta_g$) and free radical-dependent oxygenation to a much greater extent. This is the first report to demonstrate the occurrence of O_2 ($^1\Delta_g$)-specific oxygenation of unsaturated fatty acids in living animals.

Keywords: *Photoageing, lipid peroxidation, singlet oxygen, hydroperoxide, hairless mouse, TLC blotting*

Introduction

Singlet molecular oxygen [O_2 ($^1\Delta_g$)] is involved in reactive oxygen species (ROS) responsible for a variety of pathophysiological events by reacting with bioactive components directly or modulating cellular redox signalling. O_2 ($^1\Delta_g$) is highly electrophilic and therefore attacks the π-bonds of organic compounds to produce hydroperoxides or endoperoxides (*ene* reaction), although this ROS is not an oxygen radical inducing a radical chain reaction [1]. O_2 ($^1\Delta_g$)-dependent oxygenation of unsaturated fatty acids and their esters has long been known to yield an isomeric mixture of hydroperoxides [2]. The radical chain reaction of membrane lipids also yields an isomeric mixture *via* the peroxyl radical as an intermediate. Nevertheless, the hydroperoxide isomers formed by O_2 ($^1\Delta_g$)-based oxygenation differ in composition from those derived from the radical chain reaction, as the oxygenation is a non-radical reaction [3].

There seem to be three mechanisms for the generation of O_2 ($^1\Delta_g$) in biological systems. One is the self-reaction of peroxyl radicals involving a cyclic mechanism from a linear tetraoxide intermediate proposed by Russell [4]. Direct evidence for the production of O_2 ($^1\Delta_g$) from lipid peroxyl radicals was recently obtained using ^{18}O-labelled linoleic acid hydroperoxides (LA-OOH) [5,6]. The second mechanism is the reaction of hypochlorous acid (HOCl) with hydrogen peroxide in phagocytic cells [7]. The third is the Type-II reaction of photosensitized oxidation in which an excited photosensitizer transfers its

Correspondence: Junji Terao, Department of Food Science, Graduate School of Nutrition and Bioscience, The University of Tokushima, Tokushima 770-8503, Japan. Tel: +81-88-633-7087. Fax: +81-88-633-7089. Email: terao@nutr.med.tokushima-u.ac.jp

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energy to a ground state oxygen molecule to form O_2 ($^1\Delta_g$). Yasui and Sakurai [8,9] confirmed the generation of O_2 ($^1\Delta_g$) in the skin of live rodents exposed to ultraviolet light A (UVA) using chemiluminescent detection and imaging. Yamazaki et al. [10] found that the product of the O_2 ($^1\Delta_g$)-specific oxygenation of cholesterol, cholesterol 5α-hydroperoxide, accumulated in the skin of rats treated with an oral dose of a photosensitizer and subsequently exposed to visible light. We recently combined thin layer chromatography (TLC) with gas chromatography-electron ionization-mass spectrometry/selective ion monitoring (GC-EI-MS/SIM) to quantify peroxidized cholesterol and succeeded in detecting cholesterol 5α-hydroperoxide in the skin of live hairless mice [11]. However, no direct evidence of the O_2 ($^1\Delta_g$)-specific oxygenation of esterified and free unsaturated fatty acids *in vivo* was obtained.

In the present study, we aimed to detect and quantify O_2 ($^1\Delta_g$)-specific oxygenation products of two major C-18 unsaturated fatty acids, oleic acid and linoleic acid in esterified and free forms, as these peroxidized lipids seem to not only exert cytotoxic effects but also act as a photo-oxidative stress signal [12,13]. The results indicate that O_2 ($^1\Delta_g$)-dependent oxygenation of esterified oleic acid and linoleic acid occurs in the skin *in vivo*.

Materials and methods

Chemicals and reagents

Methyl oleate (methyl 9*Z*-octadecenoate) and methyl linoleate (methyl 9*Z*, 12*Z*-octadecadienoate) were purchased from Nu-Chek Prep. Inc. (Elysian, MN). Uniformly ^{13}C-labelled methyl oleate (*U*-^{13}C-methyl oleate) and uniformly ^{13}C-labelled methyl linoleate (*U*-^{13}C-methyl linoleate) were obtained from Spectra Stable Isotopes (Columbia, MD). Isomeric mixtures of methyl oleate hydroperoxides (MO-OOHs) and methyl linoleate hydroperoxides (ML-OOHs) were prepared from both labelled and unlabelled methyl oleate and methyl linoleate using methylene blue-sensitized photo-oxidation according to methods described previously [2]. The purity of MO-OOHs and ML-OOHs was checked by TLC and their concentrations were determined with a lipid hydroperoxide (LPO) assay kit (Cayman Chemical Co. Ann Arbor, MI) [14]. The extract was stored at -30°C after filtration with Milex R-HV (Millipore, Billerica, MA). Just before the GC-EI-MS/SIM analysis, MO-OOHs and ML-OOHs were converted to trimethylsilyl derivatives by reducing their hydroperoxyl groups to hydroxyl groups with $NaBH_4$ and subsequent trimethylsilylation with trimethylsilyldiazomethane as described elsewhere [2,15]. All other reagents and solvents were of guaranteed reagent grade from Kanto Chemical Co. (Tokyo, Japan).

Experiments with animals

Male Hos;HR-1 hairless mice, 8 weeks old, were purchased from SLC Japan, Inc. (Hamamatsu, Japan) and maintained under standard experimental conditions (25°C, 60% humidity; light and dark cycle every 12 h) according to the Guidelines for Animal Experimentation of the University of Tokushima. Mice were fed with standard chow (Oriental Yeast Co. Ltd., Tokyo, Japan) and water *ad libitum* for 3 weeks. For the experiment *in vivo*, mice were then exposed to irradiation with a UVA projection lamp (Matsushita Electric Industrial Co. Ltd. Osaka, Japan) for 8 h. The total amount of UVA delivered to the skin was adjusted to 47 J/cm^2. After the irradiation, mice were anaesthetized with sodium pentobarbital (50 mg/kg) and a section of dorsal skin, 1.0 cm $\times$ 2.0 cm, was obtained to avoid contamination from hypodermic fats and stored at -80°C prior to the analysis but for no more than 5 days. For the experiment *in vitro*, skin without irradiation was prepared as above and also stored at -80°C.

Preparation of skin samples for GC-EI-MS/SIM

For the experiment *in vivo*, nine volumes of 0.1 M Tris-HCl buffer (pH 7.4) was added to the dorsal skin preparation and then the mixture was homogenized with a Polytron homogenizer (Kinematica AG, Littau/luzern, Switzerland). For the experiment *in vitro*, skin homogenate was prepared as above and then exposed to UVA light at the same intensity as in the experiment *in vivo* for 8 h at 37°C. In both experiments, total lipids were immediately extracted from 1.0 ml of the homogenates by the method of Bligh and Dyer [16] after the addition of internal standard mixtures, each 5 nmol of *U*-^{13}C-MO-OOHs and *U*-^{13}C-ML-OOHs. The extract was evaporated *in vacuo* and the residual lipids were dissolved in 200 µl of methanol solution. For the reduction of the hydroperoxyl group and the oxo group of peroxidized lipids in the solution, a few crystals of $NaBH_4$ were added to the solution and kept in an ice bath for 1 h. Then a few drops of acetic acid were added and lipids were again extracted by the method of Bligh and Dyer [16] and finally the solvent was evaporated *in vacuo*. For the transmethylation of the hydroxyl derivative of esterified lipids, the residue was then mixed with 28% sodium methoxide in 600 µL of methanol solution and left at 60°C for 1 h. After a few drops of acetic acid were added to the solution, water (1.0 ml) and hexane (1.0 ml) were added successively. After centrifugation, the hexane layer was collected as the solution containing methyl esters of hydroxyl fatty acids. For the methylation of free fatty acids in the extracted lipids, the residue was dissolved into 200 µL of methanol and 30 µL of 10% trimethylsilyldiazomethane in hexane solution (Tokyo Chemical Industry Co. Ltd, Tokyo, Japan) and left at

room temperature for 30 min. After the addition of a few drops of acetic acid, the solvent was removed and a chloroform solution was added. Both solutions were charged onto a silica gel $60F_{254}$ TLC plate (0.25 mm thickness, Merck) and developed with a solvent of hexane and diethyl ether (1:1, by vol). The plate was then blotted onto a PVDF membrane as described previously [17,18]. After the blotting, the spot whose Rf value corresponded to standard hydroxyl derivatives of methyl oleate and linoleate ($Rf = 0.30 \pm 0.05$) was cut out and immersed into a solution of chloroform and methanol (1:1, by vol) with shaking for 1 min. Then, the solvent was evaporated *in vacuo* and the residue was mixed with 50 μl of *N, O*-bis (trimethylsilyl) trifluoroacetamide in acetonitrile solution and incubated at 60°C for 5 min for the preparation of trimethylsilyl derivatives. For the analysis of the fatty acid composition of skin lipids, the lipid extract obtained from non-irradiated skin homogenate was analysed with GLC after transmethylation using a fused silica capillary column, CP-Wax (0.32 mm × 30 m length × 0.2 mm thickness; Supelco, Bellefone, PA) [19].

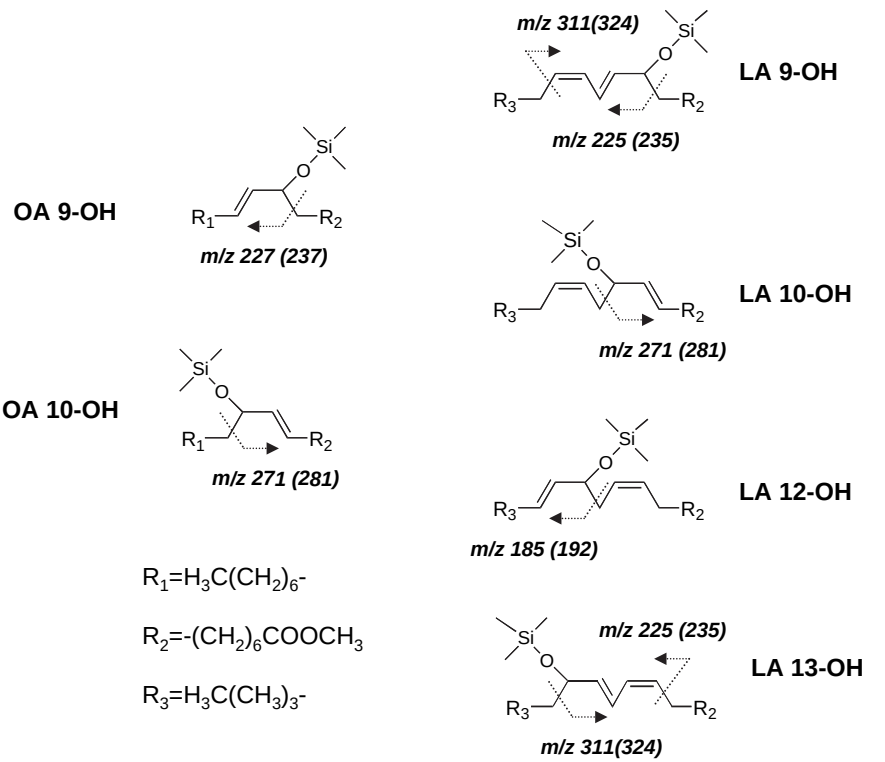
GC-EI-MS/SIM

The GC-EI-MS analysis was performed with an AQP5050 mass spectrometer (Shimadzu Co., Kyoto, Japan) equipped with a SPB-1 fused silica capillary column (30 m × 0.25 mm ID, 10 μm film thickness; Supelco, Bellefonte, PA) and splitless injection of 1 μl in the electron impact ionization mode at 70 eV [20]. The carrier gas, helium, was applied at a flow rate of 1.0 ml/min. The injection temperature was set at 260°C and the column oven was held at 50°C for 2 min before being elevated to 290°C at a rate of 10°C/min. The column oven was kept at the final temperature for 10 min. Mass spectra were obtained in the full-scan mode over a mass range from *m/z* 50 to 600. SIM was performed at a dwell time of 200 ms.

Results

GC-EI-MS/SIM of standard MO-OOHs and ML-OOHs for the quantification of peroxidized oleate and linoleate in the skin

Scheme 1 shows the fragmentation patterns of the trimethylsilyl derivatives of isomeric MO-OHs and ML-OHs in the mass spectrometric analysis. O_2 ($^1\Delta_g$)-specific oxygenation of methyl oleate is known to produce equal amounts of the 9- and 10-isomers [21,22]. The fragment ions due to α-cleavage of the trimethylsilyloxyl group are formed at *m/z* 227 for the 9-isomer and *m/z* 271 for the 10-isomer [2].



Scheme 1. Fragmentation patterns of the trimethylsilyl derivative of methyl hydroxyloctadecenoate from MO-OOHs and trimeylsilyl derivative of methyl hyroxyloctadecadienoate from ML-OOHs. Isomer-specific α-cleavage ions were obtained. Numbers (*m/z*) in parentheses are calculated from *U*-^{13}C-MO-OOHs and *U*-^{13}C-ML-OOHs. OA 9-OH; methyl 9-hydroxy-10*E*-octadecenoate; OA 10-OH: methyl 10-hydroxy-9*E*-octadenoate; LA 9-OH: methyl 9-hydroxy-10*E*,12*Z*-octadecadienoate; LA 10-OH; methyl 10-hydroxy-8*E*,12*Z*-octadecadienoate; LA 12-OH; methyl 12-hydroxy-9*Z*,13*E*-octadecadienoate; LA 13-OH; methyl 13-judroxy-9*Z*,11*E*-octadecadienoate.

For the trimethylsilyl derivatives of *U*-^{13}C-MO-OHs, characteristic fragment ions occur at *m/z* 237 and *m/z* 281 for the 9- and 10-isomers, respectively. In the case of methyl linoleate, the two unconjugated diene isomers (the 10-isomer and 12-isomer) and two conjugated diene isomers (the 9-isomer and 13-isomer) are evenly formed by O_2 ($^1\Delta_g$)-specific oxygenation [21–23]. Fragment ions at *m/z* 271 and *m/z* 185 are ascribed to α-cleavage of the unconjugated 10- and 12-isomers of the trimethylsilyl derivatives, respectively [2,20]. The fragment ions at *m/z* 281 and *m/z* 192 are also characteristic of the 10- and 12-isomers derived from *U*-^{13}C-MO-OHs. Both ions at *m/z* 225 and *m/z* 311 containing pentadiene structure are characteristic of conjugated 9- and 13-isomers [2,20]. The fragment ions at *m/z* 235 and 324 are from the conjugated isomers of *U*-^{13}C-ML-OHs similarly to unlabelled conjugated isomers. We therefore selected these fragment ions for SIM to quantify isomeric MO-OOHs and ML-OOHs in the GC-EI-MS analysis.

Figure 1 shows the result of GC-EI-MS/SIM for isomeric MO-OOHs. The fragment ions derived from the 9-isomer and 10-isomer, both labelled and unlabelled, were clearly detected in the chromatogram (Rt = 19.5 min). The standard curves with the peak area ratio (unlabelled to labelled) obtained by the mixture of unlabelled and labelled MO-OOHs were linear in both the 9- and 10-isomers ($r = 0.999$) in the range between 0.1–5.0 nmol in the sample solution (data not shown). Figure 2 also shows representative SIM chromatograms with characteristic ions for trimethylsilyl derivatives of ML-OHs. The fragment ions for the unconjugated 10- and 12-isomers both appeared at Rt = 19.3 min. In addition, the characteristic ions for the conjugated 9- and 13-isomers appeared at Rt = 19.5–19.6 min. Therefore, the 9-isomer and 13-isomer could not be well distinguished because of similar retention times and the same fragment ions in the SIM chromatogram. Thus, the sum of the peak areas arising from the 9- and 13-isomers was used for the standard curve with the mixture of labelled and unlabelled ML-OOHs for the calculation of isomeric ML-OOHs. The standard curve with the peak area ratio obtained for the mixture of unlabelled and labelled forms was linear for both the 10- and 12-isomer and the sum of the 9- and 13-isomer ($r = 0.998$, 0.999 and 0.999, respectively) in the range 0.1–2.5 nmol in the sample solution (data not shown). Collectively, it is concluded that GC-EI-MS/SIM for trimethylsilyl derivatives is capable of quantifying hydroperoxide isomers derived from oleate and linoleate in biological samples separately, when *U*-^{13}C MO-OOH and *U*-^{13}C-ML-OOH are used as internal standards and the peroxidized oleate and linoleate in esterified and free forms are converted to their methyl esters.

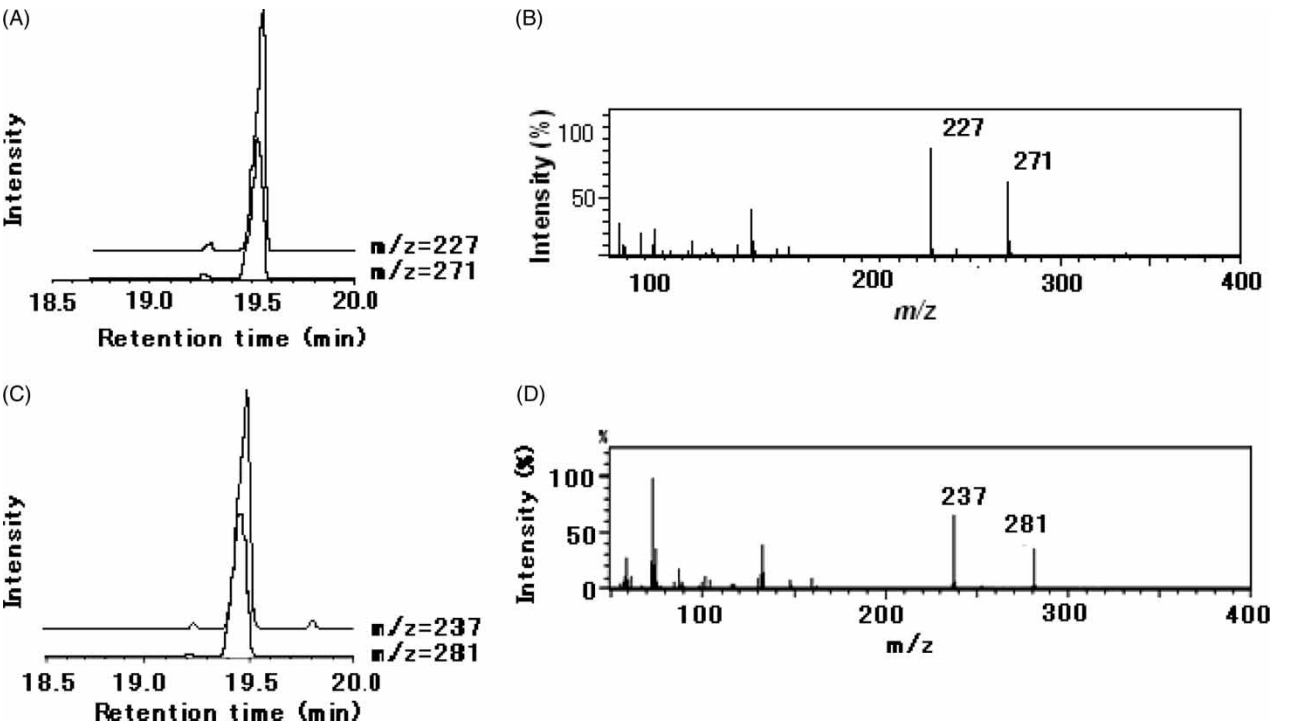


Figure 1. GC-EI-MS/SIM analysis of isomeric MO-OHs. MO-OHs were prepared by methylene blue-sensitized photo-oxidation and analysed as trimethylsilyl derivatives. (A) SIM chromatogram of unlabelled MO-OHs and (B) the mass spectrum at RT = 19.5 min. (C) SIM chromatogram of labelled *U*-^{13}C-MO-OHs and (D) the mass spectrum at Rt = 19.5 min.

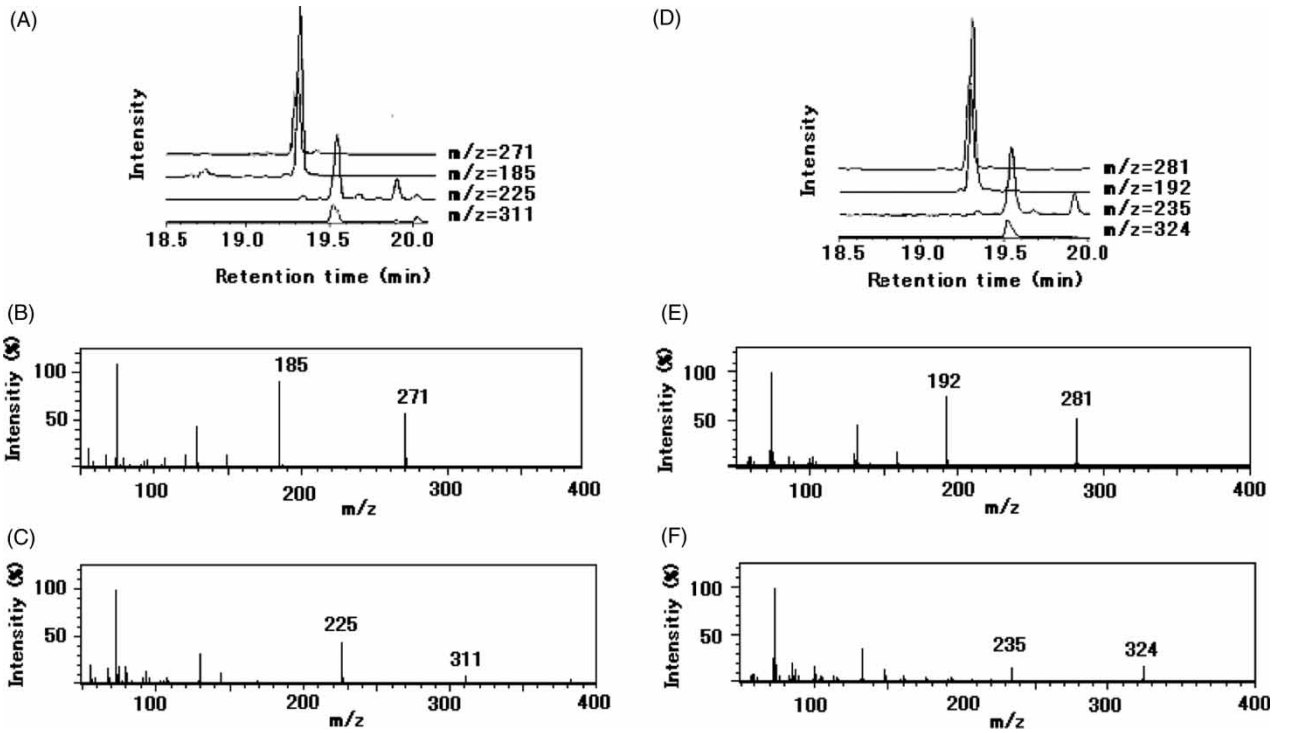


Figure 2. GC-EI-MS/SIM analysis of isomeric ML-OHs. ML-OHs were prepared by methylene blue-sensitized photo-oxidation and analysed as trimethylsilyl derivatives. (A) SIM chromatogram of unlabelled ML-OHs and (B) the mass spectrum at RT = 19.3 min. (C) mass spectrum at RT = 19.5 min. (D) SIM chromatogram of labelled *U*-^{13}C-ML-OHs and (E) the mass spectrum at Rt = 19.3 min. (F) the mass spectrum at RT = 19.5 min.

Detection and quantification of peroxidized oleate and linoleate isomers of mouse skin lipids in free form and esterified form

The fatty acid composition of mouse skin lipids determined by GLC was as follows; unsaturated fatty acids; 18:1 ($23.0 \pm 1.5\%$), 18:2 ($19.1 \pm 0.2\%$), 20:4 ($14.1 \pm 1.3\%$), 22:6 ($6.8 \pm 0.4\%$), saturated fatty acids; 16:0 ($21.8 \pm 0.7\%$), 18:0 ($11.5 \pm 0.4\%$). Therefore, oleic acid (18:1) and linoleic acid (18:2) were major C18 unsaturated fatty acids serving as the target for the analysis of peroxidized lipids. At first, peroxidized skin lipids were reduced to their hydroxyl derivatives by treatment with $NaBH_4$. Then, they were converted to methyl ester by two different methods, transmethylation with sodium methoxide for esterified oleate and linoleate and methylation of the carboxyl group with diazomethane for free oleic acid and linoleic acid. We employed TLC-blotting for isolation of the resulting hydroxyl long-chain fatty acid methyl esters. After trimethylsilylation, GC-EI-MS/SIM was applied to the trimethylsilyl derivatives as described for the standard MO-OHs and ML-OHs. Isomeric hydroxyoctadecenoic acid derived from peroxidized oleate and hydroxyoctadecadienoic acid from peroxidized linoleate were quantified successfully by applying the standard curve using labelled and unlabeled MO-OOHs and ML-OOHs. Figure 3 shows a typical SIM chromatogram for peroxidized oleate and linoleate in the esterified form obtained from live mouse skin without UVA-irradiation. The peaks for the 9-isomer and 10-isomer derived from peroxidized oleate appeared at Rt = 19.5 min (Figure 3A). The peaks for the unconjugated 10- and 12-isomers and the conjugated 9- and 13-isomers derived from peroxidized linoleate were present at Rt = 19.3 min and Rt = 19.5–19.6 min, respectively (Figure 2B). All possible isomers ascribable to O_2 ($^1\Delta_g$)-specific oxygenation of oleate and linoleate were quantified using this technique as shown in Table I. Peroxidized oleate and linoleate in esterified form were apparently predominant as compared with those in free form, indicating that lipid peroxidation mainly occurs at unsaturated fatty acids *not* in free form but in *esterified* form. The unconjugated 10- and 12-isomers in peroxidized linoleate are specific for O_2 ($^1\Delta_g$)-dependent oxygenation of linoleate, although the conjugated 9- and 13-isomers are derived from both O_2 ($^1\Delta_g$)-specific oxygenation and the free radical chain oxidation [21–23]. In addition, the 9- and 10- isomers are characteristic of O_2 ($^1\Delta_g$)-specific oxygenation of oleate [21,22]. It is therefore concluded that O_2 ($^1\Delta_g$) is, at least partly, responsible for the peroxidation of lipids occurring in the skin of live hairless mice. The fact that the sum of the conjugated 9- and 13-isomers was significantly higher than the sum of the 10- and 12-isomers in peroxidized linoleate in esterified form demonstrated that free radical-mediated lipid peroxidation proceeds concurrently

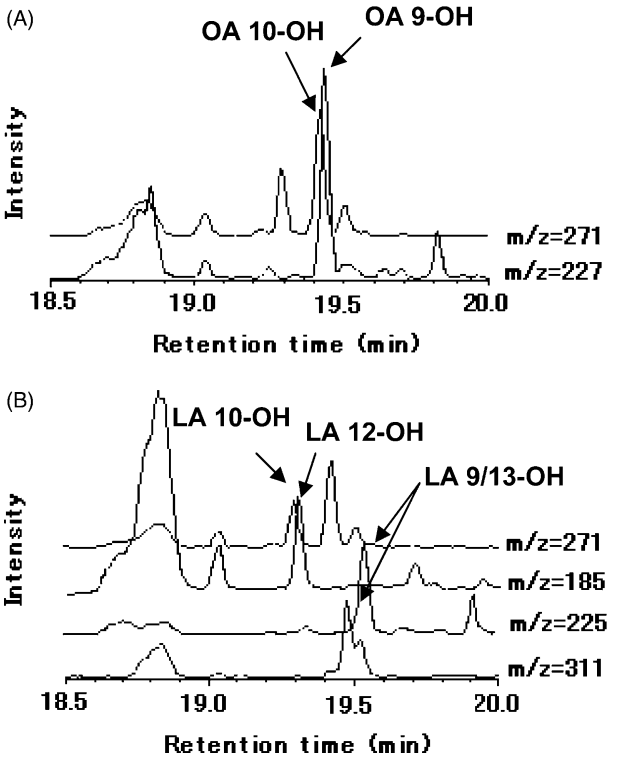


Figure 3. SIM chromatogram for peroxidized oleate and linoleate in esterified form from hairless mouse skin. Total lipids were extracted from the skin and subjected to GC-EI-MS/SIM as trimethylsilyl derivatives after reduction with $NaBH_4$ and transmethylation. SIM chromatograms were obtained by use of specific fragment ions for isomeric MO-OHs (A) and isomeric ML-OHs (B). OA 9-OH; methyl 9-hydroxy-10*E*-octadecenoate; OA 10-OH: methyl 10-hydroxy-9*E*-octadenoate; LA 9-OH: methyl 9-hydroxy-10*E*,12*Z*-octadecadienoate; LA 10-OH; methyl 10-hydroxy-8*E*,12*Z*-octadecadienoate; LA 12-OH; methyl 12-hydroxy-9*Z*,13*E*-octadecadienoate; LA 13-OH; methyl 13-hydroxy-9*Z*,11*E*-octadecadienoate.

with O_2 ($^1\Delta_g$)-dependent oxygenation and the resulting products accumulate considerably in the skin, because the free radical-mediated peroxidation of linoleate is known to produce equal amounts of the conjugated 9- and 13-isomers exclusively [3,24].

Effect of in vivo *and* in vitro *UVA-irradiation to the skin on peroxidized oleate and linoleate*

To know the difference between *in vivo* and *in vitro* lipid peroxidation occurring in UVA-exposed skin of hairless mice, GC-EI-MS/SIM was applied to skin lipid samples prepared by two different procedures. The mice were kept for 8 h with or without continuous UVA irradiation and then skin tissue was stripped and homogenized (*in vivo* study). Alternatively, homogenate was first prepared from skin tissue and then incubated for 8 h with or without continuous UVA irradiation (*in vitro* study). In both cases, the strength of the UVA was the same and lipids were immediately extracted after the irradiation. Table II shows the quantitative data for peroxidized oleate and linoleate in esterified form after 8 h with and without UVA-irradiation. In the *in vivo* experiment, little difference was observed between the amount of each isomer with UVA-irradiation and without UVA-irradiation except for that of the conjugated 10- and 12-isomers for peroxidized linoleate. Thus, it is apparent that *in vivo*, UVA-irradiation induced O_2 ($^1\Delta_g$)-driven oxygenation but did not accelerate free radical radical-mediated lipid peroxidation. In contrast, levels of all isomers were elevated significantly by *in vitro* exposure to UVA as compared with those without treatment. In particular, the conjugated 9- and 13-isomers together accounted for most of the peroxidized linoleate. Even without UVA irradiation, the sum of the 9- and 13-isomers was much greater in the *in vitro* experiment than *in vivo* experiment. Therefore, free radical-mediated lipid peroxidation occurred predominantly and the exposure to UVA accelerated free radical-driven lipid peroxidation *in vitro*.

Discussion

We already developed a method for detecting lipid hydroperoxide isomers by combining TLC blotting and GC-MS/SIM and used it to identify cholesteryl ester hydroperoxide isomers in oxidized human low density lipoprotein [20]. Here we applied the technique to a quantitative analysis of esterified and free fatty acid hydroperoxide isomers in mouse skin. For the precise quantification of peroxidized oleate and linoleate, we introduced an isomeric mixture of, respectively, *U*-^{13}C labelled hydroperoxides, *U*-^{13}C MO-OOH and *U*-^{13}C ML-OOH, as internal standards. We found neither any significant increase in MO-OOH/ML-OOH with the freezing and thawing procedure, nor the effect of non-oxidized methyl

Table I. Determination of peroxidizied oleic acid and linoleic acid in esterified form and free form as the isomers of methyl trimethylsilyl derivatives of methyl hydroxyoctadecenoate (OA-OH) and trimethylsilyl derivatives of hydroxyocdadecadienoate (LA-OH) in hairless mouse skin (nmol/g tissue).

(nmol/g tissue)	OA 9-OH	OA 10-OH	LA 10-OH	LA 12-OH	LA 9/13-OH
Esterified form	4.2±0.8	3.9±0.6	3.7±0.7	3.2±0.8	25.0±3.6
Free form	0.2±0.1	0.6±0.1	0.1±0.1	0.2±0.1	1.7±0.8

Total lipids were extracted from non-treated mice skin tissue and peroxidized oleate and linoleate were reduced by NaBH4 and concentrated by TLC blotting. Resulting hydroxy fatty acids were converted to their methyl ester and subjected to GC-EI-MS/SIM analysis as trimethylsilyl derivatives. Data were expressed as the mean value±SD ($n=5$).

Table II. Contents of the isomers of methyl hydroxyloctadecenoate from peroxidized oleate (OA-OH) and the isomers of methyl hydroxyoctadecadienoate from peroxidized linoleate (LA-OH) in esterified form obtained from hairless mouse skin with and without UVA-irradiation (nmol/g tissue). (A) *in vivo* experiment (B) *in vitro* experiment.

	OA 9-OH	OA 10-OH	LA 10-OH	LA-12-OH	LA 9/13-OH
(A) *In vivo*					
(−) UVA	6.43±1.73	6.07±1.64	3.73±1.27	3.06±1.13	23.8±5.4
(+) UVA	8.04±1.50	7.42±1.10	5.46±0.80*	5.20±0.98*	23.5±4.0
(B) *In vitro*					
(−) UVA	7.83±0.68	8.32±0.59	2.44±0.22	2.61±0.18	132.9±16.8
(+) UVA	23.05±1.39*	23.72±2.11*	10.48±1.56*	10.71±1.25*	362.6±56.3*

Total lipids were extracted from the mice skin tissue and peroxidized oleate and linoleate were reduced by $NaBH_4$ and concentrated by TLC blotting. Resulting hydroxyl fatty acids in esterified form were converted to their methyl ester and subjected to GC-EI-MS/SIM analysis as trimethylsilyl derivatives. Data were expressed as the mean value±SD ($n=5$). * Significantly different from the value obtained from the mice without UVA((−)UVA) ($p<0.05$).

linoleate added to the skin on MO-OOH/ML-OOH contents (data not shown). Thus, this methodology is suitable for determining the peroxidized lipids present in skin without interference from artificial hydroperoxides. By using GC-MS, Yoshida and Niki [25] quantified the total amount of hydroxyoctadecadienoic acid, which includes the esterified and free forms of LA-OOH and its hydroxyl derivatives in human plasma and ethythrocytes. They also suggested that the total level of hydroxyloctadecadienoic acid is a suitable oxidative stress marker *in vivo*. Nevertheless, they did not refer to the isomers of hydroxyoctadecadienoic acid derived from O_2 ($^1\Delta_g$) oxygenation. Van Kuijk et al. [26] confirmed that photo-oxidation of rat retina total lipids yielded unique unconjugated hydroperoxide isomers of arachidonoyl and docosahexanoyl esters arising from O_2 ($^1\Delta_g$) oxygenation of phospholipids. Furthermore, we found that the 9- and 10-isomers of peroxidized phosphatidylcholine resulting from the reaction of O_2 ($^1\Delta_g$) with its oleic acid moiety in mouse skin homogenate were increased by UV-A irradiation *in vitro* [19]. However, the current study demonstrated that O_2 ($^1\Delta_g$) oxygenation-specific peroxidation products, that is the 9- and 10-isomers from oleic acid and the unconjugated 10- and 12-isomers from linoleic acid, accumulate in a biological system *in vivo*. This serves as evidence that O_2 ($^1\Delta_g$) can be generated in the skin of hairless mice under normal feeding conditions. Our method did not distinguish lipid hydroperoxides from their keto/hydroxyl derivatives as the analysis was accomplished after the reduction of hydroperoxyl and oxo groups to hydroxyl groups. However, the esterified form of peroxidized fatty acid could be distinguished from the free form. Table I clearly shows the peroxidized lipids accumulated in the skin to be mostly derived from the esterified form. Our preliminary study revealed that free fatty acid makes up ~10% of the total amount of skin lipids. It is therefore suggested that for O_2 ($^1\Delta_g$) oxygenation, esterified lipids constituting the majority of skin lipids are preferred.

The distribution of isomeric hydroperoxides derived from oleic acid and linoleic acid reflects the contribution of O_2 ($^1\Delta_g$) oxygenation to the peroxidation of lipids in the skin. O_2 ($^1\Delta_g$) oxygenation of linoleic acid yields equal amounts of the 9-, 10-, 12 and 13-isomers, whereas free radical-dependent peroxidation including the radical chain reaction produces the conjugated 9- and 13-isomers exclusively [3]. The 9- and 10-isomers from oleic acid can be ascribed to the O_2 ($^1\Delta_g$) oxygenation of oleic acid, because free radical oxygenation of oleic acid producing the 8-, 9-, 10- and 11-isomers hardly occurs as compared with O_2 ($^1\Delta_g$) oxygenation [27]. Thus, O_2 ($^1\Delta_g$) oxygenation preceded free radical-dependent peroxidation in esterified oleic acid and linoleic acid in hairless mouse skin *in vivo*.

It is unclear why O_2 ($^1\Delta_g$) oxygenation occurred in the mouse skin under ordinary feeding conditions. Exposure to visible light may enhance photosensitized oxidation of Type II in the skin of hairless mice. A self-reaction of lipid peroxyl radicals may also participate in the generation of O_2 ($^1\Delta_g$) as lipid peroxyl radicals were reported to be generated in live hairless mice exposed to UV irradiation [28]. Our results clearly demonstrated that a single exposure to UVA *in vivo* does not enhance the production of hydroperoxide as compared with the exposure of skin homogenate *in vitro* (Table II). Interestingly, O_2 ($^1\Delta_g$) oxygenation also occurred significantly *in vitro*, as the unconjugated 10- and 12-isomers from linoleate and the 9- and 10-isomers from oleic acid also increased in the UVA-irradiated skin homogenate. Thus, the extent to which lipids are peroxidized *in vitro* seems to be exaggerated.

O_2 ($^1\Delta_g$) is suggested to be involved in the cross-linking of collagen by activating interstitial collagenase, that is matrix-metalloproteinases (MMPs) [29]. Furthermore, it is reported that lipid hydroperoxides participate in the expression of MMPs during photoageing [30]. Thus, O_2 ($^1\Delta_g$) oxygenation and the resulting lipid hydroperoxides may be responsible for the induction of photoageing. Further study is

warranted to know the relationship between the O_2 ($^1\Delta_g$)-mediated peroxidation of lipids and the activation of MMPs in the skin.

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