Involvement of Active Oxygen in Lipid Peroxide Radical Reaction of Epidermal Homogenate Following Ultraviolet Light Exposure

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To elucidate the radical mechanism of lipid peroxidation induced by ultraviolet light (UV) irradiation, an electron spin resonance (ESR) study was made on epidermal homogenate prepared from albino rat skin. The exposure of the homogenate to UV light resulted in an increase in lipid peroxide content, which was proportional to the time of UV exposure. Using ESR spin trapping (dimethyl-1-pyrroline-N-oxide, DMPO), the DMPO spin adduct spectrum of lipid radicals (L·) was measured following UV exposure (DMPO-L· : $a_N = 15.5$ G, $a_H = 22.7$ G), as was the spectrum of DMPO-hydroxyl radical (DMPO-OH, $a_N = a_H = 15.5$ G). In the presence of superoxide dismutase, the DMPO spin adduct spectrum of lipid radicals was found to be reduced remarkably. Therefore, it was shown that the generation of the lipid radicals partially involves superoxide anion radicals, in addition to hydroxyl radicals. In the ESR free-radical experiment, an ESR signal appeared at $g = 2.0064$ when the ESR tube filled with homogenate was exposed to UV light at −150°C. The temperature-dependent change in the ESR free radical signal of homogenate exposed to UV light was observed at temperatures varying from −150°C to room temperature. By using degassed samples, it was confirmed that oxygen is involved in the formation of the lipid peroxide radicals (LOO·) from the lipid radicals (L·). *J Invest Dermatol 97:115–119, 1991*

The skin is the region exposed to sunlight in humans. Lipid peroxide has been suspected of being the source of cellular damage induced by oxidative stress [1,2], especially by ultraviolet (UV) light [3,4]. In our earlier experiments [3,5], the topical application of free radical scavengers such as superoxide dismutase (SOD), coenzyme Q, and antioxidant vitamins (B_2, E, C) reduced the lipid peroxidation in the epidermis exposed to UV light. This suggests indirectly that free-radical reactions are involved in the lipid peroxidation process.

Free-radical generation is considered to be the central mechanism causing various metabolic abnormalities that lead to cell damage [4]. The oxygen toxicity is mediated by the formation of reactive reduced oxygen-free radicals including the superoxide anion (O_2^-) and hydroxyl radicals (·OH) [6]. Since the demonstration by Janzen and Liu [7] that 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO) is an effective spin trap, it has been used extensively for the study of free-radical metabolism. In the present paper, the electron spin resonance (ESR) spin-trapping technique was applied to detect the generation of active oxygen species in the lipid peroxidation process of an epidermal homogenate exposed to UV light.

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Abbreviations:
ESR: electron spin resonance (epr)
O_2^-: superoxide anion radical
·OH: hydroxyl radical
UV: ultraviolet light

MATERIALS AND METHODS

Lyophilized Epidermis To obtain an adequate protein concentration of epidermis for ESR study and to obtain reproducible results, the epidermis separated from ten albino rats was pooled together as a lyophilized material. Albino rats were killed by decapitation and bled completely for 20 seconds. Hairs from the animals' backs were plucked manually and the skin was excised from the back. Epidermis was separated from dermis by the stretch-scrape method and was homogenized in ten volumes of distilled water with a glass homogenizer. The homogenate was filtered through a cotton cheesecloth and was centrifuged at 600 × *g* for 10 min. The resulting supernatant was lyophilized. The lyophilized material was sealed in vial glass bottles and stored in a deep freezer (−140°C) until the analytic process was performed.

Spin-Trapping Agent The experiment was designed to detect the oxygen-free radicals generated during the exposure of epidermal homogenate to UV light, with the use of ESR spin trapping [8–10]. The spin-trap agent, 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO), was obtained from Labotec K.K. Tokyo, Japan (highly purified compound). The spin-trap DMPO used in this experiment indicates no free-radical impurity at the concentration used, as shown in Fig 2*A*. Therefore, DMPO was used directly without any purification. The exposure of DMPO itself to UV light (10 min) was examined to find out if UV photolysis had occurred. However, no ESR signal appeared from the DMPO following UV exposure (data not shown). It was confirmed that DMPO is not degraded by UV exposure under the present experimental conditions.

Ultraviolet Exposure The lyophilized material (10 mg dry weight) was dissolved in 200 μl of distilled water and mixed well by pipetting with a Pasteur pipette to make a homogeneous solution (homogenate). The radiant energy exposed to the homogenate was measured by a thermocouple instrument (Japan Spectroscopic Co.

0022-202X/91/$03.50

Ltd. AM1001). The amount of energy received was expressed as joule/m^2/min.

To determine the lipid radical (L·) and active oxygen species formed in homogenates following UV exposure, the ESR spin-trapping technique was applied. Fifteen μl of DMPO was mixed with 200 μl of homogenate. The mixed medium was taken into a 160-μl ESR quartz flat cell equipped with a screw knob (# ES-LC12, JEOL Ltd., Tokyo, Japan). The ESR flat cell was placed under an ultraviolet lamp (Toshiba Photochemical UV lamp, H400P, wavelength: 260–585 nm) at a distance of 45 cm. The UV irradiation was carried out for 2, 5, and 10 min (radiant energy: 5.6×10^3 joule/m^2/min).

To determine the free-radical reaction during UV exposure, the generation of lipid peroxide radicals (LOO·) from lipid radicals (L·) was examined using ESR radical determination. A quartz ESR-tube (5 mm in diameter) was filled with homogenate and placed in the ESR cavity. The UV irradiation was performed through a quartz lens installed in the front of the ESR cavity (ES-UV-05H UV lamp, 19×10^3 joule/m^2/min, 260–600 nm).

Electron Spin Resonance (ESR) The ESR spectra were recorded on a JEOL X-band spectrometer, model JES-3X (JEOL Ltd., Tokyo, Japan), at 100 kHz magnetic field modulation. The magnetic field was set at 3350 ± 50 G, microwave power 8 mW, amplitude 1.6×10^3, modulation amplitude 1.0 G, response 0.1 seconds, and sweep time 2 min (50 G/min).

In the case of DMPO spin-trapping determination, the ESR spectrum was recorded immediately after 10 min exposure of homogenate to UV light. For comparing signal height of ESR spin adducts, a Mn^{++} reference (MnO) enclosed in a specially designed closed glass tube with an adjustable gauged screw (# ES-DM1, JEOL Ltd., Tokyo, Japan) was installed in the ESR cavity [9,10]. Therefore, the Mn^{++} signal appeared at an almost uniform height in all of the ESR spectra.

In the case of free-radical determination, the temperature of the ESR cavity was controlled with a variable temperature controller by a liquid nitrogen flow system (JES-VT-3A). The temperature was monitored by a thermocouple. Free radicals formed in the homogenate were examined at certain desired temperatures. The g value was determined using tetracyanoquinondimethane lithium salt as a reference (TCNQ-Li, $g = 2.0026$).

Lipid Peroxide Content The lipid peroxide content was measured by the thiobarbituric acid (TBA) reaction [11]. The lipid peroxide content (TBA value) was expressed as n moles malondialdehyde/mg protein. Protein was determined by the biuret method [12].

RESULTS

Five milliliters of homogenate (0.5% protein) was placed in a Petri dish (6 cm in diameter) immersed in ice. The homogenate was exposed to UV light with 5.6×10^3 joule/m^2/min of radiant energy. As shown in Fig 1, the lipid peroxide content increased immediately after exposure and increased linearly with the amount of radiant energy. It was demonstrated that UV exposure induces a lipid peroxide (LOOH) in the epidermal homogenate. The radical reaction process of lipid peroxidation was then further examined with ESR technique.

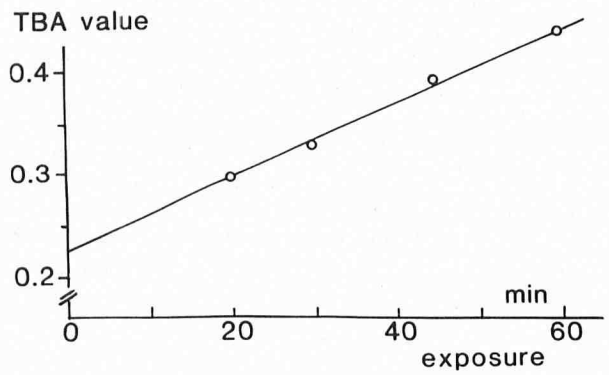

Figure 1. Effect of UV light exposure on lipid peroxide formation in epidermal homogenate. Abscissa: time of exposure to UV light (5.6×10^3 joule/m^2/min). Ordinate: lipid peroxide content (TBA value) expressed as n mol per mg protein.

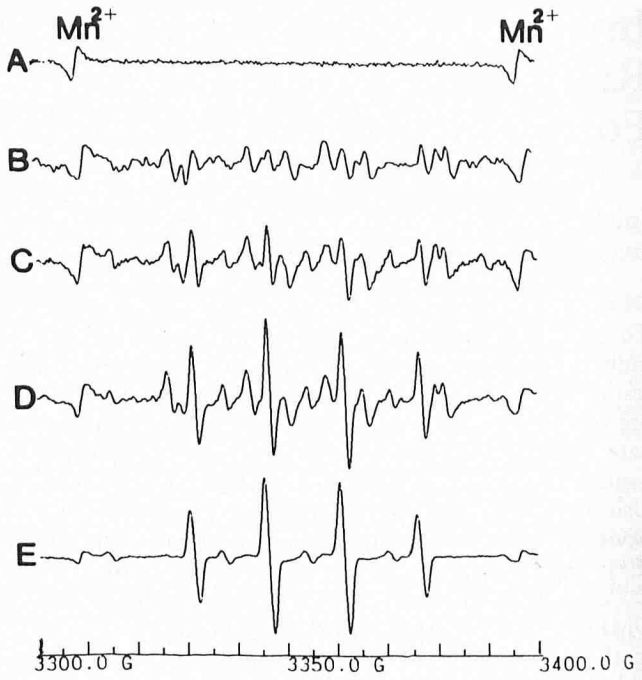

Figure 2. ESR spectra of the DMPO spin adduct obtained from epidermal homogenate exposed to UV light with different times of exposure (5.6×10^3 joule/m^2/min). *A*, DMPO only, no UV; *B*, homogenate and DMPO, UV exposure, 2 min exposure; *C*, 5 min exposure; *D*, 10 min exposure; *E*, distilled water and DMPO, UV exposure (10 min).

The ESR spectrum of the DMPO spin adduct observed in the homogenate following UV exposure is shown in Fig 2. The exposure of homogenate to UV light led to an enlargement of the ESR spectrum with increasing time of exposure. As shown in Fig 3, the ESR spectrum was analyzed to determine what kinds of DMPO-spin adducts are formed in the homogenate exposed to UV light. The sextet signal was observed with hyper-fine splittings, $a_N = 15.5$ G, $a_H = 22.7$ G, most suggestive of the trapped lipid radicals (DMPO-L·) [13,17], accompanied by a spectrum of DMPO-hydroxyl radicals (DMPO-OH, $a_N = a_H = 15.5$ G). Figure 4 indicates the increase of lipid radicals following UV exposure, being expressed as a signal intensity based on the height of the first sextet of lines relative to the Mn^{2+} signal height. It suggests that UV exposure induces the lipid radical (L·) in the epidermal homogenate proportionally to the amount of radiant energy.

In order to further determine if the ESR signal (DMPO-L·) was associated with superoxide anion (O_2^-), the experiment was performed in the presence of superoxide dismutase (SOD, superoxide anion-scavenging enzyme). Two-hundred microliters of mixed solution consisting of 160 μl of homogenate (10 mg protein), 10 μl of DMPO, and 30 μl of SOD (3000 units/ml) was exposed to UV light. As shown in Fig 5, the presence of SOD resulted in the reduction of the DMPO-spin adduct spectrum. The heat-denatured SOD left no perceptible influence on the lipid radical (Fig 5*C*).

These results suggest that generation of both O_2^- and ·OH are involved in the formation of lipid radicals (L·) in the epidermal homogenate exposed to UV.

The process of free-radical reactions from the lipid radical (L·) to peroxide radicals (LOO·) was observed in Figs 6 and 7.

As shown in Fig 6, no free-radical ESR signal was detected from the epidermal homogenate (non-UV-exposed sample) at room

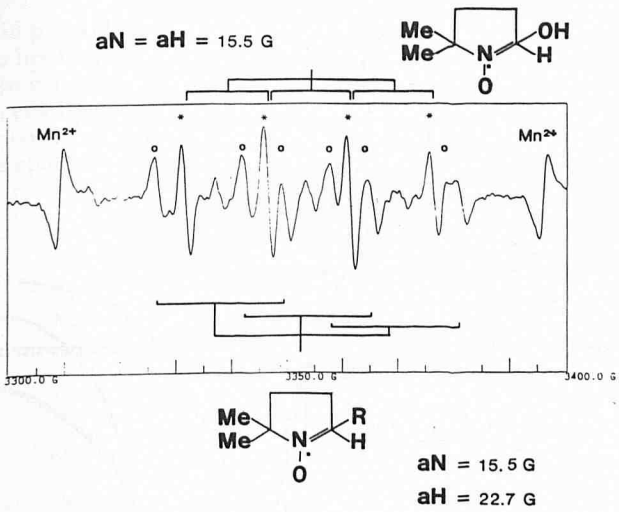

Figure 3. Analysis of ESR spin adduct spectrum obtained from epidermal homogenate following UV exposure. °, sextet signal : carbon center radical adduct (DMPO-L·); *, quartet signal : hydroxyl radical adduct (DMPO-OH). Amplitude gain = 1250, center field = 3550 G, sweep width = 50 G, modulation width = 0.80 G, microwave power = 8 mW.

temperature or even at the low temperature of −150°C. However, an ESR signal appeared at g = 2.0064 when the ESR tube filled with homogenate was exposed to UV light through the window of the cavity at −150°C. The ESR signal enlarged proportionately to the increase of radiant energy, holding the *g* value at 2.0064. The ESR signals were determined as free radicals, most suggestive of the lipid radicals (L·).

To determine the temperature-dependent change in the ESR signal, an exposure of the ESR sample tube to UV light (10-min exposure) was terminated and the cavity temperature was elevated gradually from −150°C to room temperature, using a JEOL liquid temperature controller. As shown in Fig 7, the ESR signal of the homogenate changed irregularly in shape and the *g* value changed from 2.0064 to 2.0095 at −100°C. The elevation of temperature from −100°C to −40°C changed the *g* value from 2.0095 to 2.014 and the shape of the ESR signal became sharper and smaller. The ESR signal finally diminished when the temperature was elevated to −20°C, because of molecular motion.

To determine the involvement of oxygen in the change of the ESR spectrum, anaerobic samples were prepared by degassing the air from the ESR tube enclosing the homogenate, utilizing a rotary pump (ultimate vacuum: 10^{-3} mm Hg). The process of freezing and

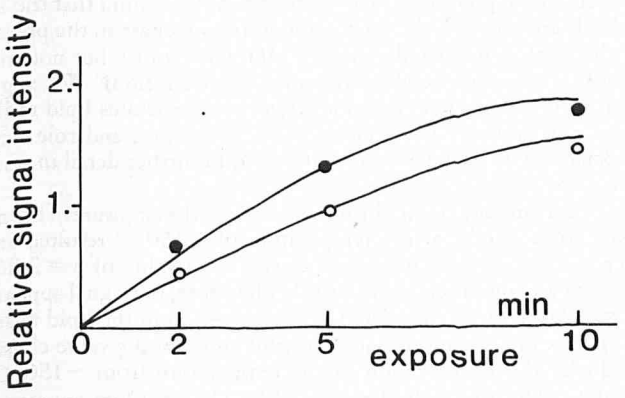

Figure 4. Effect of UV light exposure on signal intensity of lipid radical in the epidermal homogenate. The signal intensity was expressed as the height of the first sextet of lipid radical signals relative to the Mn^{2+} signal height. ● 20 mg, ○ 10 mg dry weight of lyophilized epidermis in 200 μl.

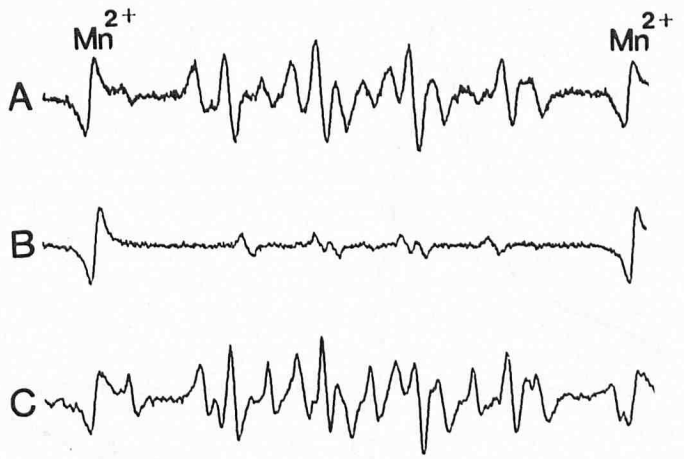

Figure 5. Effect of superoxide dismutase on the ESR spectrum of the DMPO spin adduct obtained from epidermal homogenate following UV exposure. *A*, control; *B*, SOD; *C*, heat-denatured SOD.

thawing was repeated several times. The closed degassed sample was exposed to UV light. The ESR signal appeared immediately after UV exposure at −150°C, as was observed in the control (aerobic condition). However, the change of ESR spectra following the elevation of temperature after UV exposure was quite different from that of the control. The ESR signals in the degassed sample did not change in shape and the original *g* value was maintained (Fig 8).

These results indicate that the modification of the ESR signal resulted from temperature-dependent processes associated with the oxygen.

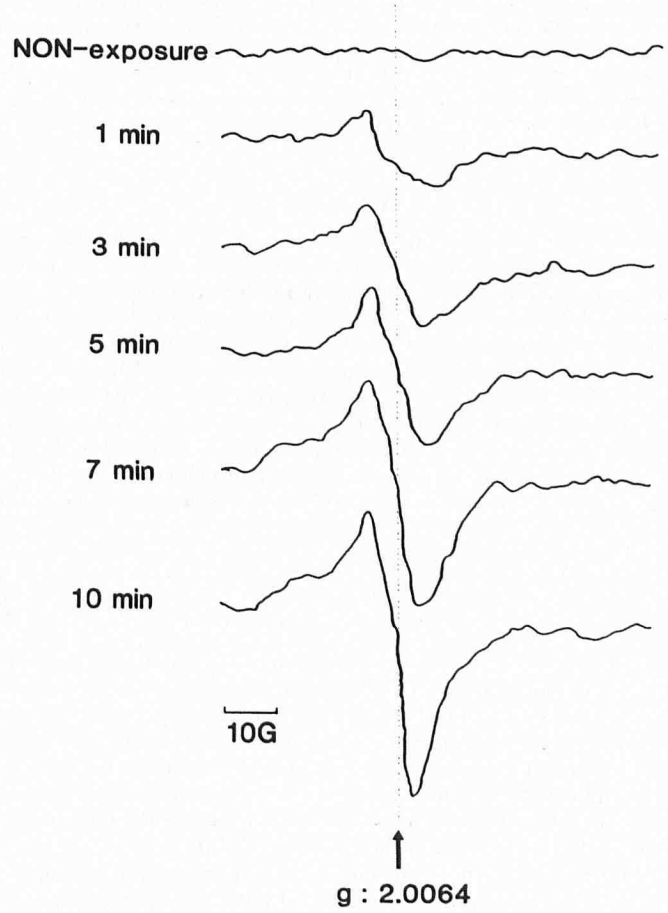

Figure 6. ESR spectra of epidermal homogenates exposed to UV light with different times of exposure. UV, 19×10^3 joule/m²/min.

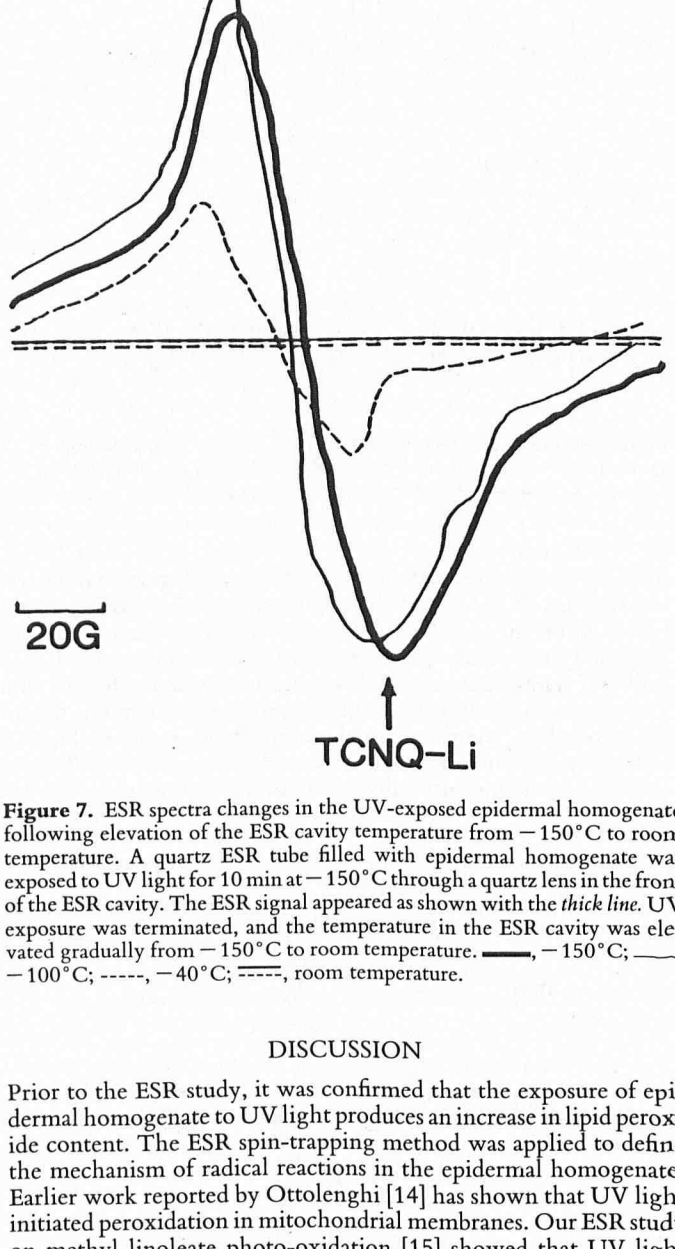

Figure 7. ESR spectra changes in the UV-exposed epidermal homogenate following elevation of the ESR cavity temperature from −150°C to room temperature. A quartz ESR tube filled with epidermal homogenate was exposed to UV light for 10 min at −150°C through a quartz lens in the front of the ESR cavity. The ESR signal appeared as shown with the *thick line*. UV exposure was terminated, and the temperature in the ESR cavity was elevated gradually from −150°C to room temperature. ▬▬, −150°C; ——, −100°C; -----, −40°C; ═══, room temperature.

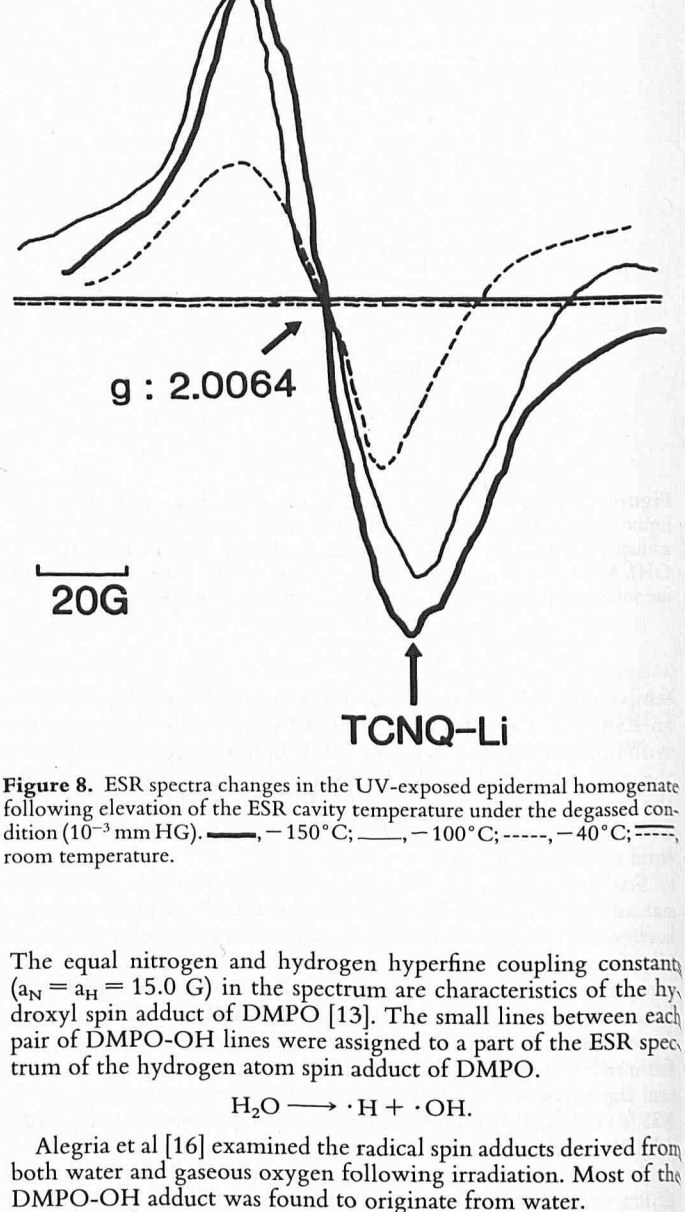

Figure 8. ESR spectra changes in the UV-exposed epidermal homogenate following elevation of the ESR cavity temperature under the degassed condition (10^{-3} mm HG). ▬▬, −150°C; ——, −100°C; -----, −40°C; ═══, room temperature.

DISCUSSION

Prior to the ESR study, it was confirmed that the exposure of epidermal homogenate to UV light produces an increase in lipid peroxide content. The ESR spin-trapping method was applied to define the mechanism of radical reactions in the epidermal homogenate. Earlier work reported by Ottolenghi [14] has shown that UV light initiated peroxidation in mitochondrial membranes. Our ESR study on methyl linoleate photo-oxidation [15] showed that UV light irradiation induces lipid peroxidation. It was suggested that organic radical formation (L·, LOO·) and oxygen are involved in the peroxidation process. The pathway from unsaturated fatty acid (LH) to UV-induced hydroperoxide (LOOH) was considered to be as follows:

$$LH \longrightarrow L\cdot + \cdot H \quad (1)$$
$$L\cdot + O_2 \longrightarrow LOO\cdot \quad (2)$$
$$LOO\cdot + L'H \longrightarrow LOOH + L'\cdot \quad (3).$$

In the present paper, the mechanism of the initial photoreaction (1) was demonstrated using ESR spin trapping. The involvement of oxygen (2) was demonstrated by the result obtained from the temperature-dependent changes on the free-radical signal.

Concerning the process of lipid radical formation, we observed that the exposure of homogenate to UV light generates hydroxyl radical (·OH) and superoxide anion (O_2^-), because the formation of lipid radical is reduced in the presence of SOD.

In order to determine the possibility of photoradical reactions of water in the homogenate, the distilled water was directly irradiated with UV light in the presence of DMPO. The ESR spectrum consisted of a 1:2:2:1 quartet following 10-min exposure (Fig 2*E*). The equal nitrogen and hydrogen hyperfine coupling constant ($a_N = a_H = 15.0$ G) in the spectrum are characteristics of the hydroxyl spin adduct of DMPO [13]. The small lines between each pair of DMPO-OH lines were assigned to a part of the ESR spectrum of the hydrogen atom spin adduct of DMPO.

$$H_2O \longrightarrow \cdot H + \cdot OH.$$

Alegria et al [16] examined the radical spin adducts derived from both water and gaseous oxygen following irradiation. Most of the DMPO-OH adduct was found to originate from water.

At the present time, it is not possible to state definitely whether it is O_2^- or ·OH that plays the major role in producing the lipid-associated radical. From the experiment of myocardial reperfusion, Zweier [17] observed that the DMPO-L· or DMPO-LOO· signal appears in the process of ·OH generation. We found that the lipid radicals appear with the incubation of homogenate in the presence of the Fenton-mediated system (·OH generation), but not in the xanthine-xanthine oxidase system (O_2^- generation). We suggest that ·OH is the direct oxygen species that generates lipid radicals (data not shown). The source of O_2^- generation and role of O_2^- associated with ·OH are to be discussed in further detail in a separate paper.

Concerning the free-radical examination, the exposure of homogenate to UV light at the temperature of −150°C resulted in an increase of free-radical content at the same value of $g = 2.0064$ without any modification of signal. Therefore, the signal appearing at $g = 2.0064$ was considered to be derived from the lipid radical (L·). It is interesting to note that ESR signal and g value changed gradually during elevation of the temperature from −150°C to −40°C. The free radical produced by UV exposure appeared to shift to other radicals. Such changes are not detected under degassed conditions. Apparently, the lipid peroxide radical is formed by being associated with oxygen ($L\cdot + O_2 \rightarrow LOO\cdot$). Finally, the

lipid peroxide radical participates in the propagation step to form the lipid peroxide ($LOO\cdot + L'H \rightarrow LOOH + L\cdot$).

In conclusion, we have provided evidence that UV exposure induces a lipid peroxide with free-radical reactions and that generation of active oxygen is involved in the formation of lipid radicals in the epidermal homogenate following UV exposure.

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