Mechanism of Lipid Radical Formation Following Exposure of Epidermal Homogenate to Ultraviolet Light

Ryohei Ogura, Masayasu Sugiyama, Juichi Nishi, and Nobuya Haramaki
Department of Medical Biochemistry, Kurume University School of Medicine, Kurume, Japan

It has been suggested that oxygen free radicals are important mediators of lipid peroxidation in the epidermis exposed to ultraviolet (UV) light. However, it is not clear whether it is the superoxide anion radical (O_2^-) or the hydroxyl radical (·OH) that plays the major role in producing the lipid radicals (L·) following UV exposure. In this study, we used electron spin resonance (ESR) technique with the spin trap (5,5-dimethyl-1-pyrroline-N-oxide [DMPO]) to determine which active oxygen species is involved in the UV-induced lipid radical formation (DMPO-L··: $a_N = 15.5$ G, $a_H = 22.7$ G). In the presence of superoxide dismutase or the metal-chelating agent, the DMPO-spin adduct spectrum of lipid radicals was reduced remarkably. The lipid radicals were formed by the hydroxyl radical generation system, not the superoxide anion generation system.

The hydroxyl radical was found to be the direct active oxygen species that can generate lipid radicals as a result of ·OH-mediated hydrogen atom abstraction. Superoxide anion radical stimulated the generation of hydroxyl radical via the iron-catalyzed reaction. *J Invest Dermatol 97:1044–1047, 1991*

Our previous studies [1–3] used electron spin resonance (ESR) spectroscopy to show that exposure of epidermal homogenate to ultraviolet (UV) light induced lipid peroxide formation via lipid radicals (L·). However, it has not been determined definitely whether it is a superoxide anion radical (O_2^-) or hydroxyl radical (·OH) that plays the direct role in producing the lipid radicals. From other results obtained from myocardial reperfusion experiments [4,5], it has been reported that alkyl radicals are observed during postischemic reperfusion and these radicals are thought to be derived from ·OH. The alkyl radicals could only be formed by ·OH-mediated hydrogen atom abstraction, and not by O_2^- alone. In our study, the ESR spin-trapping experiments were conducted to determine which oxygen radicals are involved in the generation of lipid radicals (L·) in the epidermis exposed to UV light.

MATERIALS AND METHODS

Epidermal Sample The epidermal sample prepared from pooled epidermal material was used in this study, to obtain a reproducible result [2,3]. Albino rats were killed by decapitation. 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 homogenized in 10 volumes of distilled water with a glass homogeneizer. The homogenate was filtered through a cotton cheesecloth and centrifuged at 600 × *g* for 10 min to remove cellular debris. The resulting supernatants obtained from 10 albino rats were pooled together, and the pooled supernatant was lyophilized. The lyophilized material was sealed in a glass bottle and stored in a deep freezer until the ESR experiment was performed. The lyophilized epidermis (10 mg) was dissolved in 200 μl of distilled water with a Pasteur pipette to make a homogenous solution (epidermal homogenate).

Chemicals The spin trap agent, 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from Labotec K.K., Tokyo, Japan [3,6]. Methyl linoleate (purity 95%) was purchased from Tokyo Kasei Co. Ltd., Japan, and was used without further purification [1]. Xanthine oxidase (24 units/ml) and superoxide dismutase (SOD, 3,000 units/ml) were obtained from Sigma Chemical, Co., USA.

Electron Spin Resonance (ESR) The ESR spectra were recorded with a JEOL X-band spectrometer, model JES-3X (JEOL Ltd., Tokyo, Japan) [3,6,7]. Fifteen microliters of DMPO was mixed with the sample to be analyzed (200 μl), and the mixed solution was taken into a 160-μl ESR quartz flat cell equipped with a screw knob (#ES-LC 12, JEOL Ltd., Tokyo, Japan). The ESR flat cell was put into the cavity of the ESR spectrometry. The ESR spectra were recorded at 100 KHz magnetic field modulation. The magnetic field was set at 3350 ± 50 G, microwave power 8 mW, amplitude 1250, modulation 1.0 G, response 0.1 second, sweep time 2 min (50 G/min).

Active Oxygen Generation System To determine which active oxygen species was involved in lipid radical formation, the epidermal homogenate (10 mg) was reacted directly with the generation system of active oxygen (xanthine oxidase system or Fenton reaction system) without UV exposure.

Manuscript received February 20, 1991; accepted for publication August 12, 1991.

This research was partially supported by a grant from the Shiseido Co. Ltd., Tokyo, Japan.

Reprint requests to: Dr. Ryohei Ogura, Department of Medical Biochemistry, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830, Japan.

Abbreviations:
DMPO: 5,5′-dimethyl-1-pyrroline-N-oxide
ESR: electron spin resonance (EPR)
L··: lipid radical
·OH: hydroxyl radical
O_2^-: superoxide anion radical
SOD: superoxide dismutase
UV: ultraviolet

0022-202X/91/$03.50

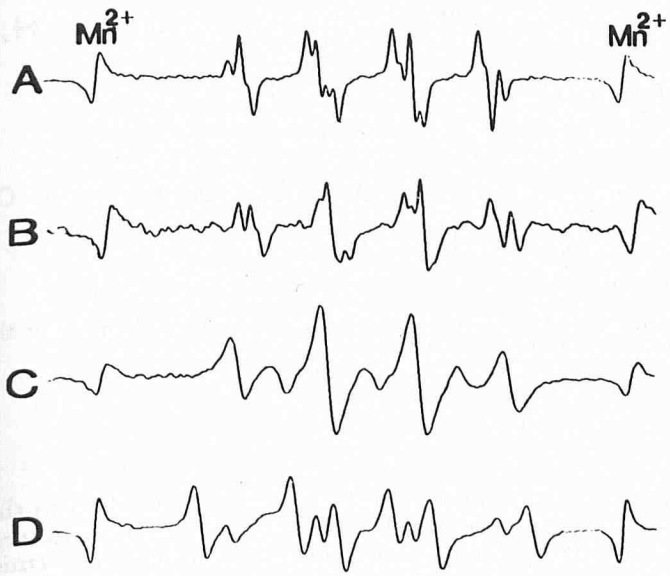

Figure 1. ESR spectrum of DMPO-spin adduct obtained from epidermal homogenate and active oxygen generation system. *A*, xanthine oxidase system alone (O_2^-); *B*, homogenate and xanthine oxidase system; *C*, Fenton reaction system alone (·OH); *D*, homogenate and Fenton reaction system.

Ninety microliters of homogenate (10 mg lyophilized epidermal sample) was mixed with the xanthine oxidase system (50 μl of 2 mM hypoxanthine, 50 μl of xanthine oxidase [0.4 units/ml] and 10 μl of DMPO [total volume in 200 μl). Beside this experiment, 130 μl of homogenate (10 mg epidermal sample) was mixed with the Fenton reaction system (20 μl of 250 mM H_2O_2, 40 μl of 100 mM $FeCl_2$ and 10 μl of DMPO).

Further additional experiments were carried out using methyl linoleate solution as a material instead of the epidermal homogenate described above. Three milliliters of 1% methyl linoleate-Folch solution (chloroform/methanol 2:1) was put into a test tube and dried to a thin film under a flow of nitrogen gas. One hundred microliters of distilled water was added to the test tube and vortexed. This methyl linoleate suspension was used.

Ultraviolet Exposure of Epidermal Homogenate Two hundred microliters of epidermal homogenate described above was mixed with 15 μl of DMPO. The mixed medium was taken into the ESR flat cell. The ESR flat cell was placed under a UV lamp (Toshiba Photochemical UV lamp, H400P, wavelength 240–585 nm) at a distance of 45 cm. The UV irradiation was carried out for 10 min (radiant energy 5.6×10^3 J/m^2/min). The ESR spectra were recorded. DMPO itself was exposed to UV light (10 min) to determine whether or not a UV photoreaction occurs. However, no ESR signal attributable to a photo-oxidized DMPO nitroxide metabolite appeared following UV exposure of DMPO itself [3]. It was confirmed that no photodegradation of DMPO occurs under the present experimental conditions.

RESULTS

The epidermal homogenate was mixed with the O_2^- generation system (xanthine oxidase system) or the ·OH generation system (Fenton reaction system), without UV exposure. Figure 1*A* shows the ESR spectrum of the xanthine oxidase system alone. The spectrum of DMPO adduct (DMPO-OOH) appears with hyperfine splitting, $a_N = 13.4$ G, $a_H^\beta = 11.2$ G, and $a_H^\gamma = 1.4$ G [8]. DMPO spin trapping confirmed that O_2^- are generated by the xanthine oxidase system. When the epidermal homogenate was added to the xanthine oxidase system, the ESR spectrum of the DMPO-OOH adduct diminished slightly in intensity as shown in Fig 1*B*; however, no spectrum of DMPO-L· appeared. Figure 1C indicates the

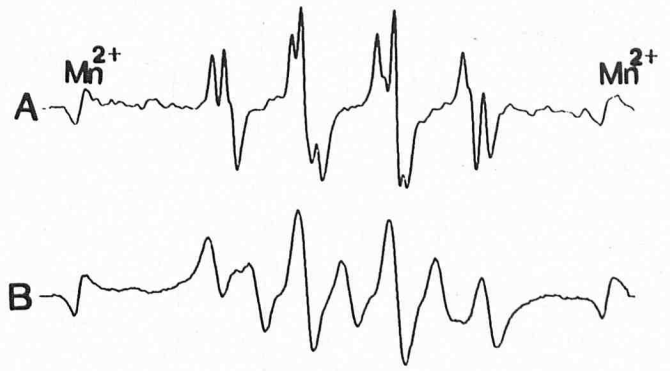

Figure 2. ESR spectrum of DMPO-spin adduct obtained from methyl linoleate. *A*, linoleate and xanthine oxidase system (O_2^-); *B*, linoleate and Fenton reaction system (·OH).

ESR spectrum obtained from the Fenton reaction system alone. The spectrum (DMPO-OH) is observed as a 1:2:2:1 quartet with hyperfine splitting, $a_N = a_H = 15.0$ G [8]. It is shown that hydroxyl radicals are generated by a Fenton reaction. When the homogenate was mixed with the Fenton reaction system, the DMPO-trapped L· was cleary identified (Fig 1*D*). The sextet ESR spectrum of DMPO-L· appeared with hyperfine splitting ($a_N = 15.5$ G, $a_H = 22.7$ G) in the presence of the Fenton reaction system.

A further experiment using another simplified system was carried out to confirm that hydroxyl radicals are a direct mediator of the lipid radical formation. Methyl linoleate was used as a material instead of epidermal homogenate. The ESR spectrum of the spin adduct shown in Fig 2*A*, which corresponds to the superoxide anion generation system, indicates no evidence of the generation of DMPO-L·. However, the spectrum of the DMPO-lipid radical adduct (DMPO-L·) appeared when the Fenton reaction system was mixed with methyl linoleate (Fig 2*B*). Therefore, the lipid radicals derived from linoleate were from the ·OH generation system (Fenton reaction), not the O_2^- generation system (xanthine oxidase system).

To find the role of a metal on the lipid radical formation under UV exposure, the homogenate was exposed to UV light in the presence of an iron-chelating agent. In the presence of 5 mM EDTA, the solution of epidermal homogenate and DMPO was exposed to UV light for 10 min. As shown in Fig 3*C*, the ESR spectrum of DMPO-L· was greatly reduced, as compared with that in the non-chelating agent sample (Fig 3*B*). It is suggested that the formation of DMPO-L· is associated with the iron-mediated reaction.

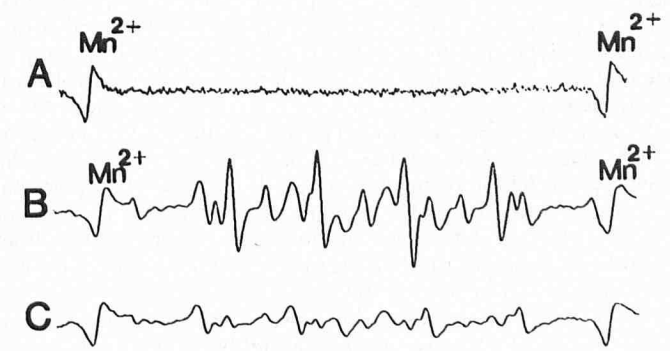

Figure 3. ESR spectrum of DMPO-spin adduct obtained from the UV-exposed epidermal homogenate in the presence of metal chelating agent. *A*, homogenate, DMPO, no UV exposure; *B*, homogenate, DMPO, and UV; *C*, homogenate, DMPO, EDTA, and UV.

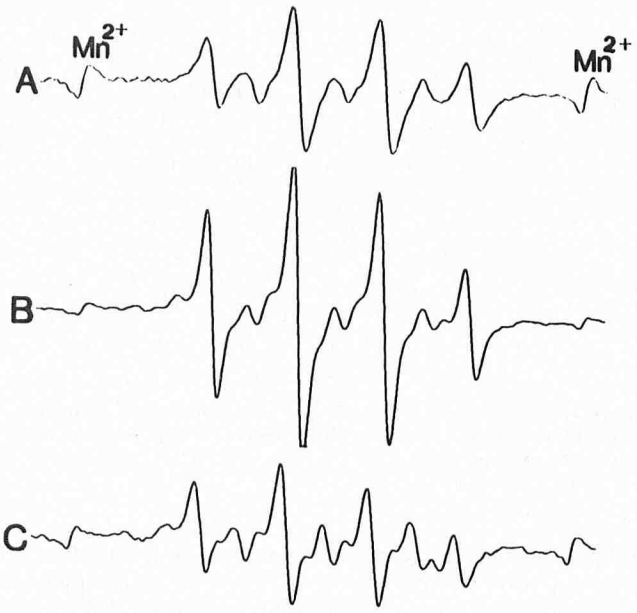

Figure 4. ESR spectrum of DMPO-spin adduct obtained from the combination of xanthine oxidase and Fenton reaction systems. *A*, Fenton reaction system alone; *B*, Fenton reaction system and xanthine oxidase system; *C*, Fenton reaction system, xanthine oxidase system, and SOD.

To determine the possible role of superoxide anion on the interaction with hydroxyl radical generation, the combination was examined without UV exposure.

As shown in Fig 4*B*, the ESR spectrum of DMPO-OH was found to increase several folds in the combined use of the Fenton reaction system and the xanthine oxidase system. The ESR peak height of DMPO-OH spectrum in the combination use showed greater intensity, as compared with that in the Fenton reaction system alone (Fig 4*A*). When superoxide dismutase (SOD) is added to the combination used, the ESR peak height of DMPO-OH spectrum was reduced to the original level of that of the Fenton reaction system alone (Fig 4*A*), as shown in Fig 4*C*.

DISCUSSION

The epidermal sample used in this study was prepared by a lyophilization process. In a previous paper [3], the ESR examination was carried out to determine whether a radical artifact was produced in the lyophilized epidermis. It was confirmed that no ESR free radical artifact was observed even in a low temperature of −150°C [3].

In our previous study [3], epidermal homogenate exposed to UV light induced lipid radicals. The DMPO-spin trapping indicated the sextet signal with hyperfine splittings ($a_N = 15.5$ G, $a_H = 22.7$ G) most suggestive of the carbon center radicals, accompanied with a spectrum of DMPO-hydroxyl radicals (DMPO-OH, $a_N = a_H = 15.5$ G) [4,8]. It was suggested that hydroxyl radicals are involved in the formation of lipid radicals following exposure of epidermis to UV light. Therefore, we investigated the role of ·OH and confirmed that the sextet signal was the same as that derived from the Folch soluble (chloroform/methanol 2:1) fraction of the epidermal homogenate exposed to UV light. Therefore, the ESR spectrum of the carbon center radical was considered to be derived from lipid radicals. In addition, the ESR signal intensity of DMPO-L· was reduced in the presence of SOD.

For the first part of our present study, experiment was conducted to determine which active oxygen species has a direct role on the generation of lipid radicals in the epidermal homogenate. The coexistence of the xanthine oxidase system with the epidermal homogenate produced no evidence of the generation of DMPO-L·.

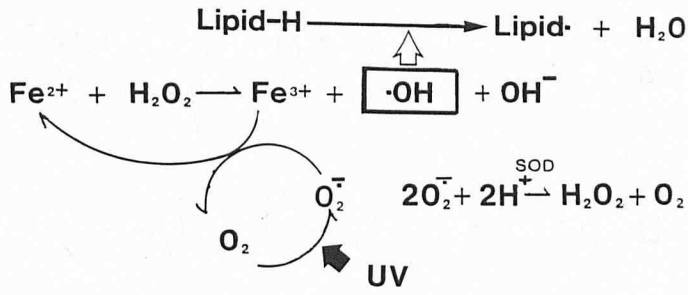

Figure 5. The proposed mechanism for generating lipid radicals in the epidermis exposed to ultraviolet light.

From the above result, it is not clear that O_2^- is not involved in the formation of the lipid radicals, because O_2^- scavenging substances, such as SOD [7] or keratin proteins [9], are present in the epidermis. We previously reported [7] that SOD activity is distributed through the whole epidermis. The SOD enzyme scavenges the O_2^- to the hydrogen peroxide H_2O_2.

Therefore, a simplified system using methyl linoleate was used in an additional study. We found that the lipid radicals appear in the methyl linoleate incubated with the ·OH generation system, but not with the O_2^- generation system. The results of both experiments imply that the hydroxyl radicals are a direct mediator of lipid radical formation, as a result of ·OH-mediated hydrogen atom abstraction.

By a myocardial reperfusion experiment, Zweier [4] also found a direct relationship between ·OH generation and lipid radicals formation. Arroyo et al [5], using 2-methyl-2-nitrosopropane spin trapping, demonstrated that ·OH initiate the lipid peroxidation of the myocytic sarcolemmal membrane.

The formation of DMPO-L· was found associated with an iron-mediated reaction. In addition, the generation of hydroxyl radicals was enhanced in the presence of xanthine oxidase system. From these results, it was suggested that the iron-mediated Fenton reaction is recycled, being coupled with the O_2^- generation system: Fe^{+++} formed by the Fenton reaction is reduced to Fe^{++} with O_2^- produced by xanthine oxidase system [11]. The O_2^- formed in a reaction medium must react rapidly with trace amounts of metals, such as iron, for recycling the Fenton reaction. In fact, the spectrum of DMPO-OOH adduct is not observed in the reaction medium with the combined presence of Fenton reaction and the xanthine oxidase system (Fig 4*B*):

$$Fe^{++} + H_2O_2 \rightarrow \cdot OH + Fe^{+++} + OH^- \text{ (Fenton reaction)},$$
$$Fe^{+++} + O_2^- \rightarrow Fe^{++} + O_2.$$

Therefore, we feel that the O_2^- must convert rapidly to the more reactive ·OH via the iron-catalyzed Fenton reaction. However, which mechanism is involved in the O_2^- generation following UV exposure will require a further study.

In our DMPO spin-trapping study, the exposure of epidermal homogenate to UV light was found to induce the DMPO-lipid radicals, accompanied with DMPO-hydroxyl radicals. In conclusion, Fig 5 shows the proposed mechanism for generating UV-induced lipid radicals. At first, the O_2^- is formed in the epidermis following exposure to UV light. The O_2^- generate ·OH via the iron-mediated reaction. The ·OH are the primary reactive oxygen species leading to the formation of the lipid radicals.

REFERENCES

1. Sugiyama M, Kajiyama K, Hidaka T, Kumano S, Ogura R: Lipid peroxidation and radical formation in methyl linoleate following ultraviolet light exposure. J Dermatol 11:455–459, 1984
2. Ogura R, Sugiyama M: Cellular membrane damage and lipid peroxide

following exposure of the skin to ultraviolet rays. In: Kligman AM, Takase Y (eds.). Cutaneous Aging. University of Tokyo Press, Japan, 1988, pp 459–471

3. Nishi J, Ogura R, Sugiyama M, Hidaka T, Kohno M: Involvement of active oxygen in lipid peroxide radical reaction of epidermal homogenate following ultraviolet light exposure. J Invest Dermatol 97:115–119, 1991
4. Zweier JL: Measurement of superoxide-derived free radicals in the reperfused heart. J Biol Chem 263:1353–1357, 1988
5. Arroyo CM, Mak IT, Weglicki WB: Spin trapping of free radicals formed during peroxidation of sarcolemmal membrane. Free Radic Res Commun 5:369–376, 1988
6. Ogura R, Ueta H, Sugiyama M, Hidaka T: Distribution of superoxide dismutase activity in the epidermis: measurement with ESR spin trapping. J Invest Dermatol 94:227–229, 1990
7. Fuchs J, Packer L: Electron paramagnetic resonance (ESR) in dermatologic research with particular reference to photodermatology. Photodermatol Photoimmunol Photomed (in press)
8. Buettner GR: Spin trapping. ESR parameter of spin adducts. Free Rad Biol Med 3:259–303, 1987
9. Matsuo I, Yoshino K, Ohkido M: Mechanism of skin surface lipid peroxidation. In: Seiji M, Bernstein IA (eds.). Normal and Abnormal Epidermal Differentiation. University of Tokyo Press, Japan, 1983, pp 135–143
10. Canada AT, Werkman RF, Mansbach II CM, Rosen GM: Biochemical changes in the intestine associated with anoxia and reoxygenation: in vivo and in vitro studies. J Free Rad Biol Med 2:327–334, 1986
11. Minotti G, Aust SD: The requirement for iron (III) in the initiation of lipid peroxidation by iron (II) and hydrogen peroxides. J Biol Chem 25:1098–1104, 1987