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RAPID COMMUNICATION

ULTRAVIOLET LIGHT-INDUCED FREE RADICAL FORMATION IN SKIN: AN ELECTRON PARAMAGNETIC RESONANCE STUDY

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ABSTRACT-It has been suggested that ultraviolet light induces free radical formation in skin, leading to photoaging and cancer. We have demonstrated by electron paramagnetic resonance that the ascorbate free radical is naturally present in unexposed skin at a very low steady state level. When a section of SKH-1 hairless mouse skin in an EPR cavity is exposed to UV light (4,500 J $m^{-2} \cdot s^{-1}$, Xe lamp, 305 nm cutoff and IR filters), the ascorbate free radical signal intensity increases. These results indicate that UV light increases free radical oxidative stress, consistent with ascorbate's role as the terminal, small-molecule antioxidant. The initial radicals produced by UV light would have very short lifetimes at room temperature; thus, we have applied EPR spin trapping techniques to detect these radicals. Using α-[4-pyridyl 1-oxide]-N-*tert*-butyl nitrone (POBN), we have for the first time spin trapped a UV light-produced carbon-centered free radical from intact skin. The EPR spectra exhibited hyperfine splittings that are characteristic of POBN/alkyl radicals, a^N = 15.56 G and a^H = 2.70 G, possibly generated from membrane lipids as a result of β-scission of lipid alkoxyl radicals. Iron can act as a catalyst for free radical oxidative reactions; chronic exposure of skin to UV radiation causes increased iron deposition. Using our spin trapping system, we have shown that topical application of the iron-chelator, Desferal, to a section of skin reduces the UV light-induced POBN adduct radical signal. These results provide direct evidence for free radical generation and a role for iron in UV light-induced dermatopathology. We suggest that iron chelators can serve as photoprotective agents by preventing these oxidations.

INTRODUCTION

Depletion of stratospheric ozone makes the upper atmosphere more transparent to ultraviolet (UV) light. Skin is a very susceptible target organ to UV radiation; exposure can lead to premature aging and carcinogenesis.[1,2] There is considerable circumstantial evidence suggesting that UV light-induced damage in skin is due to free radical generation.[3] However, direct detection of these putative free radicals is difficult due to their high reactivity and low steady-state concentrations; thus, the majority of the evidence is circuitous.[4-6]

The only method currently available to directly detect free radicals is electron paramagnetic resonance (EPR). The ascorbate free radical is the only radical directly detectable by EPR in skin at room temperature.[7] The initial radicals produced by UV light would have very short lifetimes at room temperature making them undetectable, thus EPR spin trapping techniques have been applied. Spin trapping involves the addition reaction of a highly reactive radical to a diamagnetic compound (spin trap), forming a long-lived free radical product (spin adduct), which is EPR detectable.[8,9] Spin trapping techniques have been used to study UV light-induced free radical production in skin homogenates.[10-13] Although these experiments indicate that UV light produces free radicals, using homogenized tissue can yield artifacts.[14] Thus, in our study pieces of mouse skin, epidermis and dermis intact, were used.

Chronic exposure of SKH-1 mouse skin to UV radiation has been found to significantly increase basal amounts of non-hemoglobin iron.[15] The presence of this excess iron could lead to increased free radical oxidative injury.[16] Therefore application

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of metal chelators, which render iron catalytically inactive, should prevent iron-mediated free radical production and consequently reduce oxidative damage. Indeed, topical application of iron chelators to mouse skin has been found to be photoprotective by delaying the onset of UV light-induced skin tumor formation during chronic exposure.[15] In the present study, we investigate UV light-induced radicals in intact mouse skin and examine the effects of the metal chelator Desferal on this free radical production.

MATERIALS AND METHODS

Whole skin harvested from SKH-1 hairless male mice (Charles River Laboratories, Portage, MI) was cut into EPR usable pieces (≅ 0.7 cm x 1.0 cm, epidermis and dermis), placed in a Wilmad Glass Co. (Buena, NJ) tissue cell, and positioned in the EPR cavity. EPR spectra were obtained at room temperature using a Bruker ESP 300 spectrometer (Bruker Instruments; Karlsruhe, Germany), operating at 9.73 GHz with 100-kHz modulation frequency. The EPR spectrometer settings for the ascorbate radical experiments were: microwave power, 40 milliwatts; modulation amplitude, 0.66 G; time constant 0.3 s; scan rate 8 G/41.9 s; receiver gain, 2 x 10^6. The epidermal surface of the skin was exposed to UV light while in the EPR cavity. The light source was a Photomax 150 W xenon arc lamp (Oriel Corporation, Stratford, CT); wavelengths below 300 nm were filtered out using a Schott WG 305 filter (Duryea, PA). Infrared radiation from the light was removed by a 5 cm water filter. The filtered light fluence rate, including the visible wavelengths, as measured using a Yellow Springs Instrument (Yellow Springs, OH) model 65A radiometer with a 6551 probe, was ≅ 4,500 J/m^2·s, assuming the cavity grid transmits 75% of the incident light.

For the spin trapping experiments, α-[4-pyridyl 1-oxide]-N-*tert*-butyl nitrone (POBN) and Desferal were obtained from Sigma Chemical Co., St. Louis, MO. A 250 mM POBN aqueous stock solution was prepared immediately prior to use. No increase in background EPR signal occurred when POBN alone was exposed to UV light. In the spin trapping experiments, skin sections were placed on ice in weigh boats and 100 µL of 250 mM POBN was applied to the epidermis for ten minutes. In the experiments using Desferal, 100 µL of a 10 mM stock was applied to skin for ten minutes and blotted off prior to the application of the spin trap. The skin samples were lightly blotted after incubation with spin trap and placed in a Wilmad tissue cell. The epidermal surface was exposed to UV light while in the EPR cavity, using the same light source set up as in the ascorbate radical experiments. EPR spectra were obtained at room temperature using a Bruker ESP 300 spectrometer, operating at 9.73 GHz with 100-kHz modulation frequency. EPR instrument settings for the spin trapping experiments were: microwave power, 40 milliwatts; modulation amplitude, 0.75 G; time constant 0.3 s; scan rate 60 G/41.9 s; receiver gain, 1 x 10^6.

RESULTS AND DISCUSSION

Using room temperature EPR, a very low steady-state level of the ascorbate free radical ($Asc^{\bullet-}$) was observed to be naturally present in skin (Fig. 1, inset). Due to ascorbate's role as the terminal small molecule antioxidant,[17,18] the ascorbate radical concentration can be used as a marker of oxidative stress.[18] Exposure of mouse skin to UV light, while in the EPR cavity, results in an increase in the ascorbate radical signal height indicating that during UV exposure the skin is undergoing free radical oxidative stress (Fig. 1).

The resonance-stabilized ascorbate radical is easily detectable in skin by EPR. However, the non-resonance stabilized radicals initially produced by UV light would have very short lifetimes at room temperature; thus, EPR spin trapping techniques have been applied. Previous EPR spin trapping studies on UV light-induced free radicals in skin have employed skin homogenates.[10-13] In our studies of UV light-induced radicals we have used pieces of mouse skin, epidermis and dermis intact. When skin, which had been incubated with the spin trap POBN, is exposed to UV light while in the EPR cavity, a carbon-centered POBN spin adduct, as well as the ascorbate radical is observed (Fig. 2). The spin adduct EPR spectra exhibit hyperfine splittings that

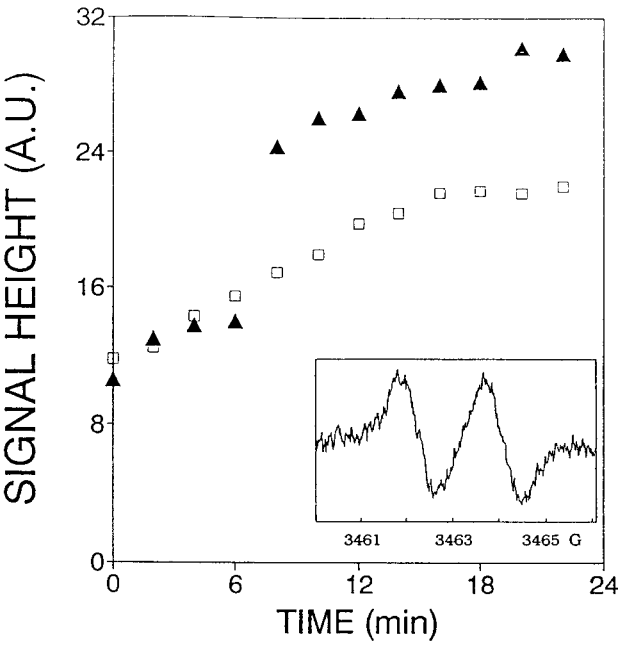

FIGURE 1. Ascorbate Radical Signal in Skin Exposed to UV Light. □, $Asc^{\bullet-}$ EPR signal intensity in skin with no UV light exposure. Δ, $Asc^{\bullet-}$ EPR signal intensity during UV light exposure. The epidermal surface of the skin was exposed while in the cavity to UV light after collection of the fourth data point. Each data point represents the mean of three separate experiments. After UV light exposure, a paired comparison of each curve showed them to be statistically different, $p < 0.001$.

Inset: Ascorbate Radical Doublet EPR Signal in Mouse Skin. Each line of the ascorbate radical doublet is actually a triplet of doublets. However, our instrument settings were selected for maximum sensitivity; thus, only a doublet with $a^{H4} \cong 1.8$ G is observed.

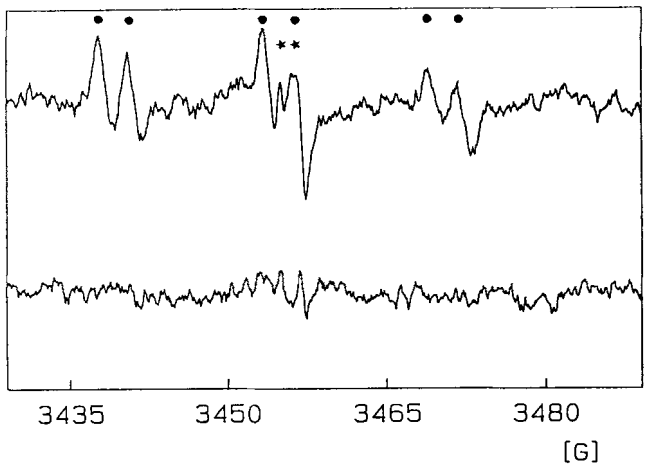

FIGURE 2. POBN Spin Trapping of a Radical from UV Light Exposed Mouse Skin. All EPR measurements were made at room temperature. The POBN adduct of a carbon-centered (•), a^N = 15.56 G, a^H = 2.70 G, as well as the ascorbate free radical, a^{H4} ≡ 1.8 G (☆), are shown. The lower spectrum is from skin exposed to room light only, the upper spectrum is from skin exposed to the UV light source.

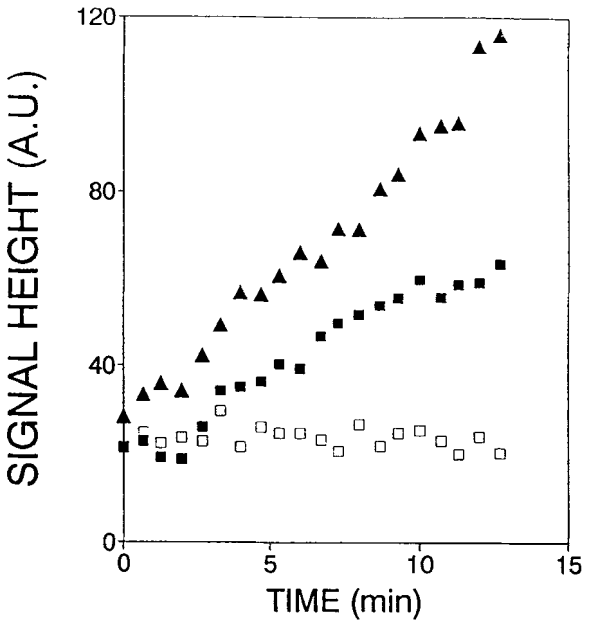

FIGURE 3. POBN Spin Trapping of a Radical Signal in Mouse Skin, Treated with Desferal Prior to UV Light Exposure. □, POBN spin adduct signal intensity from untreated skin that was exposed to room light only. Δ, POBN spin adduct signal intensity from untreated skin that was exposed to UV light. ■, POBN spin adduct signal intensity from skin treated with Desferal and exposed to UV light. In the UV light studies, the skin was exposed to the UV source after collection of the fourth data point. The signal height of the first doublet of the POBN adduct was used for all measurements. Each data point represents the median value of three separate experiments. After UV light exposure, a paired comparison of each curve showed them to be statistically different, $p < 0.001$.

are characteristic of POBN/alkyl radicals, a^N = 15.56 G and a^H = 2.70 G, possibly generated from membrane lipids as a result of β-scission of lipid alkoxyl radicals.[17,19] Because POBN itself may be photochemically active, as a control, we examined a photochemical system containing POBN and a linoleic acid emulsion (both with and without 10 μM iron). No POBN spin adducts were observed, indicating that it is unlikely that our results in skin are due to POBN photochemistry. To our knowledge our spin trapping result in skin is the first direct observation of the apparent formation of lipid-derived free radicals produced in intact skin by UV light exposure.

Evidence for UV light-induced lipid peroxidation has been obtained previously using skin homogenate systems.[10,11] Lipid peroxidation is considered to play a major part in the pathogenesis of UV light-induced damage.[20,21] In skin, approximately 25% of the total surface lipids are unsaturated;[22,23] poly-unsaturated lipids are the vulnerable targets in lipid peroxidation.[17] Iron acts as a catalyst in lipid peroxidative reactions. It reacts with lipid hydroperoxides producing lipid alkoxyl radicals, LO•, in a Fenton-type reaction.

$$LOOH + Fe(II) \text{ ----------> } LO^{\bullet} + HO^{-} + Fe(III)$$

These energetic alkoxyl radicals can then participate in the initiation of further chain propagation reactions generating additional lipid and lipid peroxyl radicals or they can undergo β-scission forming aldehydes and short chain alkyl radicals, such as ethyl and pentyl radicals.[17,19] We believe that in our study the spin trapped radical is a short chain alkyl species, based on the hyperfine splitting constants.

Skin is a significant site of iron excretion; 0.24 - 0.6 mg is lost daily by epithelial cell desquamation, as well as by gland and sweat secretions.[24] Chronic exposure of skin to UV radiation has been found to cause a significant increase in the basal amounts of non-hemoglobin iron.[15] The presence of this excess iron could lead to increased free radical oxidative injury.[16] Indeed, topical application of iron chelators to skin, which render iron catalytically unreactive, has been found to be photoprotective by delaying the onset of UV light-induced skin tumor formation during chronic exposure.[15] In our study, the topical application of the iron chelator Desferal to skin prior to UV light exposure significantly reduced the lipid radical EPR signal (Fig. 3). Desferal has no significant UV absorption at wavelengths > 280 nm. Therefore, its mechanism for protection cannot be simply due to blocking the UV light. Our results indicate that iron is involved in UV light-induced free radical formation in skin.

The use of iron chelators to reduce free radical formation is an entirely new approach to

photoprotection and subsequently skin cancer prevention. Instead of only blocking the UV radiation from being absorbed by skin, as is done now with UV absorbers, such as *para*-aminobenzoic acid and cinnamate (common ingredients found in over-the-counter sunscreens), skin could also be protected by preventing propagation of iron-catalyzed free radical damage. UV light is implicated as the leading cause of skin cancer; depletion of the earth's natural sunscreen, the ozone layer, is predicted to escalate skin cancer incidence. Thus, the development of this photoprotective concept is imperative.

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