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RAPID COMMUNICATIONS

ESR DETECTION OF ENDOGENOUS ASCORBATE FREE RADICAL IN MOUSE SKIN: ENHANCEMENT OF RADICAL PRODUCTION DURING UV IRRADIATION FOLLOWING TOPICAL APPLICATION OF CHLORPROMAZINE

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Abstract - Using electron spin resonance spectroscopy, we observed that UV radiation (330 nm) increased the endogenous ascorbate free radical concentration in hairless mouse (HRS/J) skin. When the skin was topically treated with a chlorpromazine solution prior to illumination, UV irradiation caused the ascorbate free radical concentration to increase even more. This observation suggests that there is an increased UV-induced oxidative stress in the presence of chlorpromazine, probably caused by the production of free radicals from chlorpromazine.

INTRODUCTION

The UV wavelengths of sunlight are known to produce deleterious effects on skin, ranging from sunburn to cancer. These effects have often been attributed to oxidation by free radical intermediates (Slater, 1972). Protection against UV damage by endogenous GSH[§] has recently been demonstrated with *in vivo* human fibroblasts by Tyrrell and Pidoux (1986); the level of protection approaches that afforded by excision repair. These researchers propose that the observed protection results from the antioxidant properties of GSH.

The ascorbate anion (AH^-), an antioxidant which is naturally present in tissue, reacts rapidly with the glutathiyl free radical yielding GSH and $A^{\bullet -}$ with a rate constant of 6×10^8 $dm^3 mol^{-1} s^{-1}$ at pH 7 (Forni *et al.*, 1983). Thus, ascorbate may serve as an important component in the "repair" of UV radiation-produced free radicals.

Chlorpromazine often induces phototoxic and photoallergic reactions (Fitzpatrick et al., 1963). This toxicity has been attributed to the formation of radicals by the UV radiation-induced dechlorination of CPZ (Jose, 1979). Using the ESR spin trapping technique, Motten *et al.* (1985) have clearly demonstrated the photoproduction of the dechlorination radical from CPZ. Moreover, the dechlorination radical abstracts hydrogen atoms from a variety of substrates as effectively as the hydroxyl or phenyl radicals.

The object of the present work was to determine whether ESR spectroscopy could be used to detect UV radiation-induced free radicals in skin treated with CPZ. While direct evidence for CPZ radicals was not obtained, indirect evidence for CPZ-produced radical reactions in skin was found by monitoring the UV radiation-induced increase in concentration of the ascorbate radical derived from endogenous ascorbic acid. We believe that this is the first report of $A^{\bullet -}$ being observed by ESR in skin tissue.

MATERIALS AND METHODS

Skin from HRS/J female mice 6-8 weeks old, was obtained after killing the animals by cervical dislocation. Excess fat was removed by gentle scraping. The skin was then placed in pH 7.0, 50 mM phosphate buffer with or without 2 mM CPZ for 10 minutes, rinsed with buffer solution, then kept at ice temperature prior to ESR examination. The skin was placed in an ESR tissue cell and in the ESR spectrometer as soon as possible.

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§Abbreviations: $A^{\bullet -}$, ascorbate free radical; AH^-, ascorbate anion; CPZ, chlorpromazine; ESR, electron spin resonance; GSH, glutathione; TEMPO, 2,2,6,6-tetramethyl-piperidine-1-oxyl.

ESR spectra were recorded at 9.5 GHz with a Varian E-109 spectrometer equipped with a TM_{110} cavity. Skin samples were at room temperature during ESR measurements. Samples were irradiated at 330 nm (10 bandpass nm) in the cavity with a Schoeffel 1000 W Xe arc lamp and monochromator combination. The irradiance was approximately 15 W/m^2, assuming the cavity grid transmits 75% of the incident light, as measured with a Yellow Springs Instruments Model 65 radiometer.

Because the ascorbate signal is weak and decays rapidly it is essential to minimize the time between harvesting the skin and collecting the spectra. Each tissue sample required different tuning of the ESR spectrometer and thus, without accurate knowledge of both the magnetic field strength and the operating-frequency of the spectrometer for a particular tuning condition, it was often difficult and time-consuming to find the very weak signal of the ascorbate free radical. To minimize this time, a small piece of plastic tubing with a speck of solid spin label, TEMPO (g = 2.0062), sealed in the end, was lowered into the cavity just to the point where its signal could be found with the spectrometer's field sweep option. The TEMPO signal was centered by adjusting the magnetic field strength, the TEMPO was then removed and after adjusting the instrument settings, the ascorbate free radical signal (g = 2.0052) was monitored. The identity of the ascorbate free radical was confirmed by comparing the intrinsic signal with that obtained after applying pure sodium ascorbate to the skin sample.

RESULTS AND DISCUSSION

We observed the ESR signal of the ascorbate free radical in freshly prepared hairless mouse skin. The intensity of the signal decreased with time. When the skin sample was irradiated with 330 nm UV light, the intensity of the ascorbate free radical signal increased by 25-35% under the experimental conditions used (Fig. 1). If the light intensity was increased, the signal intensity of $A^{\bullet -}$ also increased.

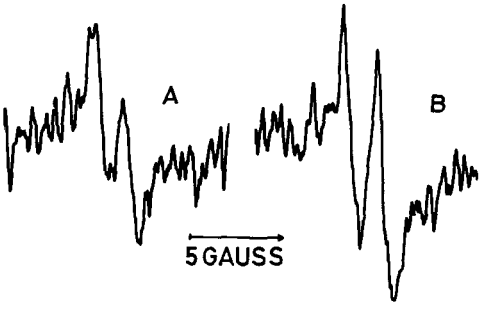

Figure 1. Ascorbate free radical observed in hairless (HRS/J) mouse skin: A. without UV radiation; B. with 330 nm UV radiation. The observed hyperfine splitting is 1.77 gauss, quite similar to other reported values for $A^{\bullet -}$ (Fisher and Hellwege, 1977-1979), and in addition, features consistent with an additional hyperfine splitting of approximately 0.2 gauss from two equivalent hydrogens can be seen, although the signal to noise is too low and the modulation amplitude too high to make a positive assignment. Instrument settings were: Mod. Amp., 1 gauss; Gain, 3.2×10^5; Power, 20 mW; Scan, 1.33 gauss/min; Time Constant, 4 s.

When the skin samples were pretreated with CPZ prior to ESR examination, the ascorbate free radical was also observed prior to UV exposure. However, when the pretreated samples were irradiated using the same conditions as for the untreated skin, the intensity of the $A^{\bullet -}$ signal increased by a factor of two (Fig. 2).

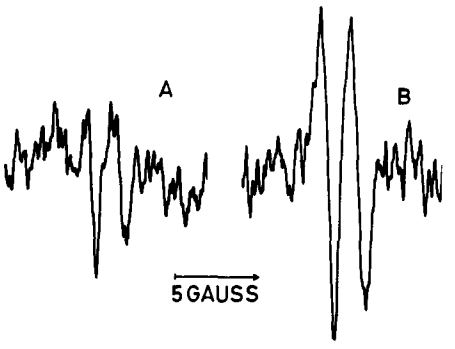

Figure 2. Ascorbate free radical in hairless (HRS/J) mouse skin treated with 2 mM CPZ aqueous solution applied topically and rinsed prior to ESR measurements: A. without UV light; B. with 330 nm UV light. Hyperfine splittings and instrument settings are as in Figure 1.

To show that 330 nm light does not affect ascorbate itself, we irradiated an aqueous solution of 1 mM ascorbate and observed no change in the ESR intensity of $A^{\bullet -}$. However, when 0.3 mM CPZ was included, the $A^{\bullet -}$ signal increased by approximately 250% (Fig. 3).

Although free radicals have been implicated in the damage produced by UV irradiation of skin, there are few reports of direct ESR detection of radical species produced in skin. Norris (1962) was not able to identify the radical he observed in UV irradiated human skin samples. Pathak (1967) observed free radical signals in human skin which he assigned to the various melanins. His samples were collected as many as four hours after death. Ogura's group concluded that the radical they observed on exposure of hairless mouse skin

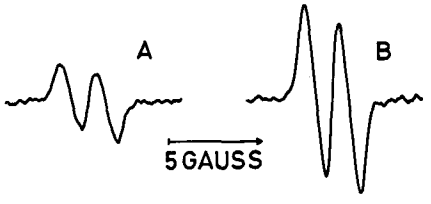

Figure 3. Ascorbate free radical in aqueous solution. Samples contained 1 mM ascorbate and 0.3 mM CPZ in pH 7.0 air-saturated 50 mM phosphate buffer: A. without UV radiation; B. with 330 nm radiation. When ascorbate was irradiated at 330 nm in the absence of CPZ, no increase in the ascorbate free radical signal was observed (not shown). Hyperfine splittings are as in Fig. 1. Instrument settings: Mod. Amp., 1 gauss; Gain 1.25 x 10^4; Power, 20 mW; Scan, 5 gauss/min; Time Constant, 1 s.

to UV light resulted from lipid peroxide radicals (Sugiyama et al., 1984a; Ogura, 1982; Sugiyama et al., 1984b). The short lag-time between the death of the animal and the ESR observation of the skin sample in our experiments allowed us to detect the weak and transient $A^{\bullet-}$ radical. Our observation of an increase in $A^{\bullet-}$ in UV radiation-exposed skin is supporting evidence for UV radiation-induced free radical processes. The larger increase in the $A^{\bullet-}$ concentration observed in CPZ-treated skin also suggests these free radical processes are enhanced by CPZ, a drug that is well known to produce free radicals upon exposure to UV radiation (Motten et al., 1985; Buettner et al., 1986; Chignell et al., 1985).

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