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Long-term follow-up study of changes in lipid peroxide levels and the activity of superoxide dismutase, catalase and glutathione peroxidase in mouse skin after acute and chronic UV irradiation

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Abstract. Lipid peroxide levels, the activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px), and the development of tanning in the skin of C57 BL/6 mice were assessed for long periods, from very early to late stages, after acute or chronic UVB irradiation. Acute UVB irradiation produced an increase in lipid peroxide levels that peaked 18 h after irradiation, after which the levels declined to a minimum 2–3 days after irradiation and then gradually rose to baseline. Chronic irradiation caused the lipid peroxide level to fall to a minimum at 0.5–1.0 weeks, after which it gradually returned to baseline by the third week. SOD and GSH-Px activities decreased sharply after acute irradiation, reaching a minimum 18 h after irradiation. Following chronic irradiation, these enzyme levels peaked after 0.5 weeks, and thereafter declined gradually to the original levels 3 weeks after irradiation. In contrast, catalase activity did not change significantly. Tanning began to increase at 1.5 weeks after irradiation, with an accelerated rate of increase from the third week. Although UVB has been reported only to decrease or impair reactive oxygen species (ROS) scavenging enzyme activity, we postulate the following from our results: (1) the increase in lipid peroxide levels observed after irradiation was due to UVB-induced ROS; (2) the parallel decrease in enzyme activities may have been due to inactivation by ROS; (3) the decrease in lipid peroxide levels following the peak at 18–h resulted from the scavenging effect of increasing SOD and GSH-Px activities, and (4) the increase in these two enzyme activities was the result of their induction by the increased lipid peroxides or ROS. In addition, these results seem to suggest a possible correlation of melanogenesis with UVB-induced ROS.

Key words: UVB irradiation – Tanning – Reactive oxygen species – Lipid peroxides – Superoxide dismutase

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Recent attention has been directed toward reactive oxygen species (ROS) and lipid peroxides, which are produced from unsaturated fatty acids in the presence of ROS, as potent inflammatory and chemical mediators capable of causing serious tissue injury and closely associated with the pathogenesis of a variety of diseases including cancer [16, 41], chronic inflammatory or degenerative diseases and aging [5, 8, 21, 23, 25, 27, 28, 30, 43]. In addition, we have found ROS to be implicated in the pathogenesis of several inflammatory dermatological diseases [29, 30].

Several reports [6, 7, 10, 22, 24, 36, 38] have indicated that, in addition to an increase in lipid peroxide levels after UV irradiation [22, 24], UV irradiation (UVB, UVA, PUVA) decreases or impairs the activity of ROS scavenging enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px). In addition, and more importantly, we have recently found using serial measurements that these enzyme activities are increased following their initial decrease after UVB radiation. The discrepancy between our results and those of others is due to the different experimental procedures and durations of study used. Many studies reported to date [6, 7, 10, 22, 24, 36, 38] were performed after only a single acute UV irradiation, and the parameters were determined for very short periods after irradiation.

In this study, changes in the levels of lipid peroxides and the activities of ROS scavenging enzymes, SOD, catalase and GSH-Px were determined serially in the skin of C57 BL/6 mice from the start to 4 weeks after acute and chronic UVB irradiation.

Despite a previous report suggesting a decrease in or an impairment of the activity of enzymes by UV-produced ROS [6, 7, 10, 24, 36, 38], the results of our study indicate a definite, although somewhat complex, relationship between lipid peroxides (or the decreased activities of SOD and GSH-Px), and the induction of (increase in) enzyme activities following the decrease.

It is well known that sunburn and sun-tan is pathogenetically related to UVB radiation, which induces the formation of $^1O_2$ [3, 35], an extremely potent ROS [2, 34]. In addition, it has been widely speculated that ROS formation is involved in the stimulation of melanogenesis

or carcinogenesis in the skin by UVB irradiation [3]. However, a correlation between the induction of sun-tan or melanogenesis and ROS has not yet been demonstrated directly. From the changes observed in this study occurring concurrently with increasing tanning in UVB-irradiated mice, we discuss the possible correlation of ROS with melanogenesis.

Materials and methods

Animals and ultraviolet radiation source

Inbred C57 black female mice (C57BL/6NCrj; Charles River Japan Inc., Japan) were used at 8 weeks of age. The shaved dorsal skin of the trunk was subjected to UVB irradiation (290–320 nm) from seven FL20SE lamps (Toshiba Electric Co., Japan) at a dose of 100 or 200 mJ/cm^2 per irradiation. The dose of 200 mJ/cm^2 was approximately half that required to induce mild oedema with a single exposure. The energy of the UVB dose was measured with a UV radiometer (UVX-31 and UVX digital radiometers; UVB Inc., USA) with a spectral responsivity of 310 nm.

Two irradiation protocols were used: acute and chronic. Mice subject to acute UVB irradiation were given a single dose, and the parameters under consideration were determined at intervals for 180 h after irradiation. In the chronic protocol, mice were exposed to repeated UVB irradiation, and the parameters were followed over a period of 4 weeks.

Measurements of the same parameters at the same intervals were carried out on age- and sex-matched control mice of the same strain in which the dorsal skin was shaved but not irradiated.

Acute irradiation with UVB

A total of 55 mice were used in this study. The hair on the back of each mouse was removed with electric clippers followed by shaving with an electric razor 24 h before UV irradiation. The shaved skin of the mice was exposed to a single dose of 200 mJ/cm^2 UVB irradiation. The animals were then sacrificed by cervical dislocation after 0, 1, 3, 6, 12, 18, 24, 48 and 72 h or 1 week. Five mice were examined at each time-point. Five mice out of 55 were sacrificed after shaving without any UVB irradiation as controls.

Chronic irradiation with UVB

A total of 125 mice were used in this study. The dorsal trunk was shaved three times per week as described above. The mice were subjected to UVB irradiation for 2 days per week at a dose of 100 mJ/cm^2. The animals were sacrified 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 weeks after the initial irradiation. At each time-point 13 mice were studied. Eight mice out of 125, shaved but not irradiated, served as controls.

Tissue preparation

The dorsal skin which had been exposed to UV radiation was carefully dissected from the underlying panniculus. Each sample was thoroughly washed and then diluted ten-fold with physiological saline and stored at −70 °C. The frozen tissue was homogenized in a freezer-mill homogenizer (6700 Freezer/Mill; SPEX industries Inc., USA) for approximately 2 min. The samples were then sonicated with a sonifier cell disrupter (W-200P; Branson Sonic Power Company, USA) at 30 W for 30 s, and then suspended in either 0.9% NaCl for lipid peroxide assay or 126 m*M* phosphate buffer for assay of SOD, catalase and GSH-Px activities.

Assay of lipid peroxide and enzyme activity

Lipid peroxide levels were estimated by determining thiobarbituric acid (TBA)-reacting substances by the method of Ohkawa et al. [33] with some modifications [30]. The standard assay mixture contained 0.1 ml 10% tissue homogenate, 0.4 ml H_2O, and 0.2 ml 7% sodium dodecylsulphate. This mixture was gently stirred and 2 ml 0.1 *N* HCl was added followed by 0.3 ml 10% phosphotungstic acid, and the samples were then left for 5 min at room temperature. Next, 1 ml of a 1:1 mixture of 0.67% TBA and acetic acid was added, and the mixture heated for 45 min at 95 °C. After cooling in ice, 5 ml *n*-butanol was added to extract the lipid peroxides, and the mixture was vortexed and centrifuged for 15 min at 1250 *g*. The lipid peroxide concentration in the butanol layer was then determined with a fluorescence spectrophotometer (MPF4; Hitachi, Japan) at 515 nm for excitation and 553 nm for emission.

SOD activity was assayed by the method previously described for skin tissue [30]. Briefly, 5% tissue homogenate was adjusted to 0.08% by the addition of Triton X-100, kept on ice for 1 h and centrifuged for 10 min at 7000 *g*; To 2 ml of the assay mixture of a xanthine–xanthine oxidase O_2^--generating system [30] was added 0.5 ml supernatant. In this system, the formation of O_2^- is determined by ferricytochrome *c* (type III) reduction, and the absorbance is measured at 550 nm using a spectrophotometer (U-3200; Hitachi, Japan). The amount of SOD in the sample required to inhibit the rate of reduction of cytochrome *c* by 50% is defined as one unit of activity, and expressed as U/mg protein.

In the SOD activity assay, the samples not only inhibited cytochrome *c* reduction but also directly reduced some of the cytochrome *c* substrate without mediation by O_2^-. Therefore, the values of SOD activity induced by the organ homogenates themselves were corrected for the organ homogenates themselves were corrected for the amount of cytochrome *c* directly reduced by the tissue homogenate. The corrected SOD activity was obtained from the following expression [30]:

$$\text{SOD activity} = \frac{a - (b - c)}{0.5a}$$

where *a* is the absorbance obtained with xanthine oxidase alone, *b* is the absorbance obtained with skin homogenate in the presence of xanthine oxidase and *c* is the absorbance obtained with skin homogenate alone before the addition of xanthine oxidase.

Catalase activity was determined by the reduction velocity of H_2O_2 in the presence of catalase-containing skin samples from 12 s to 30 s, using a spectrophotometer (U-3200; Hitachi, Japan) at 240 nm [1, 40]. The activity was determined from the following expression:

$$K = \frac{2.3}{18} \times \log \frac{A_1}{A_2}$$

where A_1 is the OD (240 nm) at 12 s in the assay mixture consisting of 10 m*M* H_2O_2 dissolved in 3 ml 50 m*M* phosphate buffer (pH 7.0) and containing 0.1 ml skin sample, and A_2 is the OD (240 nm) at 30 s in the above assay mixture.

To assess possible instability of H_2O_2 used as a substrate in the catalase activity assay, we substituted sodium perborate for the H_2O_2 substrate, according to the method of Thomson et al. [40]. Thus 0.002–0.05 ml of sample was suspended in 2.8 ml 0.05 *M* potassium phosphate buffer (pH 7.4) and preincubated at 30 °C for 5 min. The reaction was then started in the cuvette by adding 0.2 *N* $NaBO_3$ solution (neutralized at pH 9.4 with 85% H_3PO_4), and then the absorbance was recorded for 2–3 min at 220 nm.

GSH-Px activity was measured by the method of Lawrence and Burk [18], in which GSH-Px activity is coupled to the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was followed spectrophotometrically at 340 nm and 37 °C. The reaction mixture consisted of 50 m*M* potassium phosphate buffer (pH 7.0), 1 m*M* glutathione, 2 U glutathione reductase, and 1.5 m*M* cumene hydroperoxide or 10 m*M* *t*-butyl hydroperoxide. The total

volume of the mixture was made to 2.0 ml. The unit of enzymatic activity was expressed as nanomoles of NADPH oxidized per minute. Protein was measured using the method of Lowry et al. [19]. Specific enzyme activity was expressed as units per milligram of protein.

The degree of tanning was measured using a colour meter (Minolta Co. Ltd., Osaka, Japan) which measured the light reflected from tanned skin, and had the following specification: spectral sensitivity, an approximate chromatic function (CIE 1931); measurement diameter, 8 mm; repeatability chromaticity (XY), within 0.0002; colour difference (ΔE ab), within 0.07; and instrumental error (ΔE ab), within 0.6.

Results are expressed as mean ±SEM of each group (n = 5, 13 and 8). Statistical significance was determined using Student's t-test.

Results

Acute UVB irradiation

As shown in Fig. 1, lipid peroxide levels increased and peaked 18 h after irradiation ($P < 0.01$), and thereafter began to decrease. In contrast, SOD, catalase and GSH-Px activities first decreased, reaching minimum levels 4 h after irradiation (SOD, $P < 0.001$; catalase, $0.01 < P < 0.05$; GSH-Px, $P < 0.001$), and then began to increase, reaching a peak around 20–30 h after irradiation. Thereafter, GSH-Px activity remained elevated, whereas the other two activities declined toward baseline.

Chronic UVB irradiation

Lipid peroxides decreased reaching a minimum level ($P < 0.001$) between 0.5 and 1 week after irradiation (Fig. 2). Thereafter, lipid peroxides began to increase, and returned almost to the original level 3 weeks after irradiation.

In contrast, SOD and GSH-Px activities increased, peaking ($P < 0.01$) between 0.5 and 1 week after irradiation (Fig. 2). These two enzyme activities then

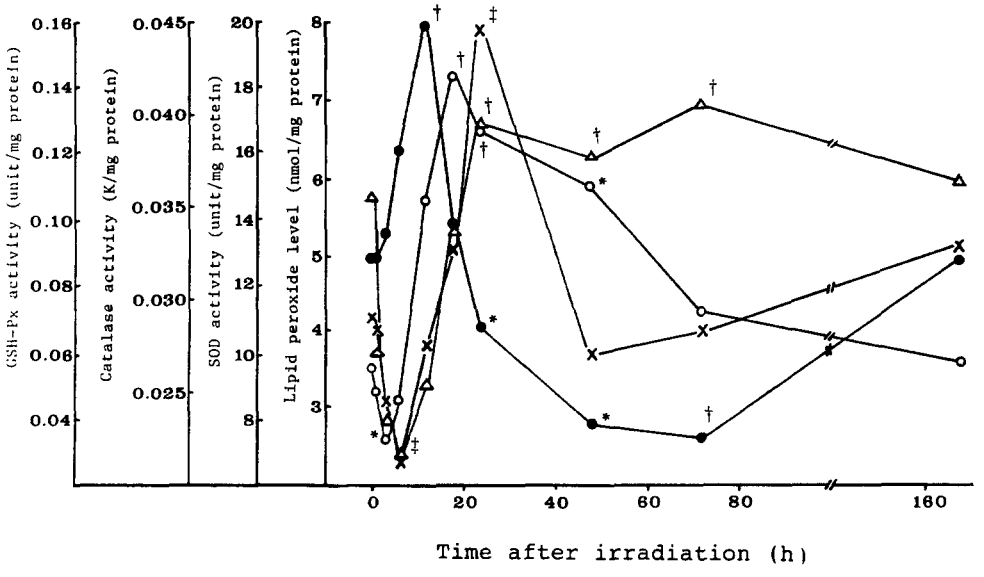

Fig. 1. Changes in lipid peroxide level and activities of SOD, catalase and GSH-Px in the skin of mice after receiving acute UVB irradiation. ●, lipid peroxide level; ○, SOD activity; ×, catalase activity; △, GSH-Px activity. * $0.01 < P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$ vs. control (before irradiation). SEM of the lipid peroxide levels and enzyme activities were between 12 and 18% of each value and are omitted for clarity

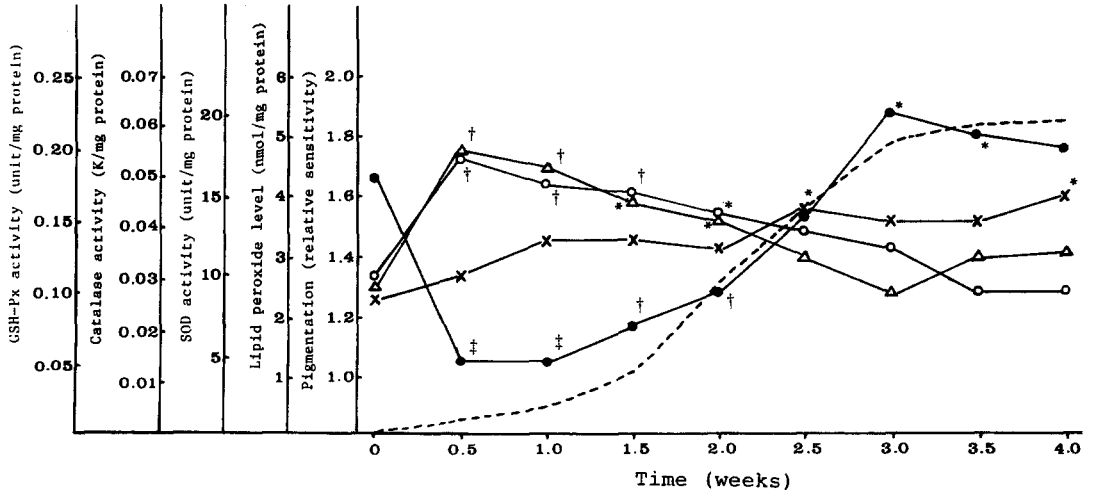

Fig. 2. Changes in lipid peroxide levels, SOD, catalase and GSH-Px activity, and pigmentation (– – –) in the skin of mice after receiving chronic UVB irradiation. – – –, pigmentation; all other symbols have the same meaning as in Fig. 1

gradually decreased, returning almost to the original levels 3 weeks after irradiation and remaining constant thereafter. Of the three scavenging activities, catalase showed the least change.

Pigmentation gradually increased for 1.5 weeks after the beginning of irradiation, and then showed an accelerated rate of increase 2–3 weeks after irradiation (Fig. 2).

When sodium perborate rather than H_2O_2 was used as the substrate in the catalase activity assay, similar results were obtained (data not shown). Thus, instability of H_2O_2 in the assay was negligible.

Non-irradiated control mice did not show any significant changes in lipid peroxide levels or the activity of any enzyme throughout the experiments, compared with the values before shaving (data not shown).

Discussion

It is widely accepted that irradiation with visible light produces $^1O_2$, the most potent ROS, in the presence of protoporphyrin and haematoporphyrin which are endogenous photosensitizers. Near-ultraviolet radiation, which is able to reach the ground and is mainly in the range 290–400 nm, produces $^1O_2$ at all sites in plants and animals since various substances such as riboflavin, vitamin K, NADH and variant tRNA all act as photosensitizers in this wavelength range [35]. UVB radiation, mainly in the range 290–320 nm, also produces $^1O_2$ in the presence of such substances as riboflavin, tryptophan, and *N*-formyl nucleorenin, and damages the eye and skin. In this connection, UVB irradiation is generally considered to produce $^1O_2$ at sites where it is irradiated.

In the present study, an increase in lipid peroxides levels was not found in chronically irradiated mice until after they had received UVB irradiation at 3.5 day intervals for 3 weeks. However, lipid peroxides levels were increased 3 to 18 h after acute UVB irradiation with a higher dose (200 mJ/cm^2). This is consistent with the results of other studies [3, 24], and seems to be due to the ROS-producing effect of UVB radiation and the resultant formation of lipid peroxides from unsaturated fatty acids. Lipid peroxides are thus considered to increase during the initial period after UVB irradiation, then to decrease, then gradually to return to normal levels, and then to increase further above baseline after 3–3.5 weeks.

With regard to changes in scavenging enzyme activities, all three enzyme activities initially decreased following a single dose of radiation, returned to normal, and then reached supranormal levels. In the case of SOD and GSH-Px activities, the elevation persisted for the duration of the experiment (180 h). In the mice treated with UVB radiation at 100 mJ/cm^2 twice per week, SOD and GSH-Px activities increased after the first dose, and then gradually decreased to the original levels.

From these findings, we postulate that the marked increase in lipid peroxides at 18 h resulted from ROS generated by UVB radiation, and that the subsequent decrease in lipid peroxide levels may have been due to scavenging by the increasing SOD and GSH-Px activities. Furthermore, the increases in SOD and GSH-Px activities after irradiation were probably due to enzymatic induction by the UVB-produced ROS. Exposure of bacteria or plants under oxidative stress induces an increase in SOD activity up to ten times that without oxygen stress [9, 37]. We have reported that erosive or ulcerative skin lesions with increased lipid peroxide levels and decreased SOD activity do not respond to treatment, while those with an increase in both lipid peroxide levels and SOD activity are easily and promptly healed [26, 30]. In addition, our group have demonstrated, as an anti-oxidative self-defence mechanism, the occurrence of similar ROS-scavenging enzyme induction with oxidative injuries in various tissues and organs [12–15, 31].

The initial decrease in enzyme activities observed after acute irradiation (Fig. 1) was most likely due to the enzyme inactivating activity of ROS induced by UVB radiation, since O_2^- has been shown to inactivate catalase [17] and GSH-Px [4], and H_2O_2 degrades SOD [11].

There are several reports [3, 6, 7, 10, 22, 24, 36, 38, 39] on the changes in free radicals, lipid peroxides and scavenging enzymes after UV irradiation. Meffert et al. [22] have demonstrated an increase in lipid peroxides in human skin after UV irradiation, and an elevation in elderly individuals. This is consistent with the results of the present study and our finding that ROS scavenging enzyme activity decreases with age [31, 32].

Although there have been many reports on the changes in ROS scavenging enzyme activities following UV irradiation, including the study of Meffert et al. which indicates a decrease in G-6-PD, most of them only reported a decrease or impairment of enzymatic activities. In contrast to the study reported here, the previous studies assessed enzymatic activities after a single exposure to UV radiation, and only followed the activities for a short period, and not from the very early to late stages after UV irradiation as performed in this study, in which induction following the decrease in enzymatic activity was observed. We propose the following mechanism: during the early stages after UVB irradiation, the antioxidant defence system in the surface of exposed skin is impaired. This seems to be due to the enzyme-denaturing action of free radicals produced after UV irradiation as previously reported [4, 11, 17]. In addition, this study demonstrated that, after a considerable decrease in enzymatic activities, an increase in their activities occurred. Thus induction of enzyme activities can be observed with long-term follow-up assays after acute or chronic UV irradiation. Our hypothesis is supported by the findings of previous studies in plants and bacteria [9, 37]. ROS-scavenging enzymes, especially SOD, are markedly induced in cells or tissues under conditions of oxidative stress.

It is noteworthy that in previous studies [24, 36] the activity particularly of SOD among the three enzymes did not increase, but rather decreased after UV irradiation. It is likely that in the previous studies, sufficient ROS was generated to denature the SOD present. Because the SOD induction capacity is lower in rodents

[15, 20] than in humans [30, 31] or bacteria, and because ROS scavenging enzymes (especially SOD) are susceptible to inactivation by ROS [4, 11, 17], an apparent lack of SOD induction may be found in rodent experiments [15, 42] when enzyme denaturing by ROS exceeds the enzyme induction capacity.

In comparison with SOD and GSH-Px activities, catalase activity was less prominently induced by chronic UVB irradiation. Unlike SOD and GSH-Px, catalase activity may be produced constitutively and be present in mouse skin at a level sufficient to scavenge whatever H_2O_2 is produced, as described by Maral et al. for erythrocytes [20]. Thus the H_2O_2 level may not have risen sufficiently to induce increased catalase activity. Consistent with this hypothesis is our previous observation that catalase is not among the ROS-scavenging enzymes induced in human leukocytes by incubation with paraquat [31, 32].

It is also of interest to note the correlation between the changes in pigmentation, lipid peroxide levels and enzyme values after chronic UV irradiation. During the first 2 weeks after chronic UV irradiation when the formation of lipid peroxides was low and oxidative toxicity was weak owing to the strong induction of each enzyme activity, the accumulation of pigmentation did not show a marked increase. In contrast, in the later stages, after 2 or 3 weeks, pigmentation began to show a gradual and marked increase in parallel with both a gradual decrease in each enzyme activity and an increase in lipid peroxide levels resulting from oxidative stress due to UV irradiation.

However, since melanin has been reported to have an ROS-scavenging effect [34], it is likely that the decrease in each enzyme activity is substituted by ROS-scavenging melanin activity. In any case, oxidative stress due to chronic UV irradiation seems to have exceeded the ROS-scavenging activity by enzymes and melanin during the later stage (at 3 and 3.5 weeks after UV irradiation).

In conflict with the results of the present study, Tegner [39] reported that tissue anoxia prevents skin inflammation and pigmentation caused by UVA irradiation but not by UVB irradiation or PUVA, suggesting that UVB irradiation does not correlate with pigmentation. This discrepancy seems to be due to the different method of UV irradiation (dose, duration, etc.) used.

Taking together the effects of UVB irradiation in B6 mice on lipid peroxide levels, SOD and GSH-Px activities, and pigmentation found in this study, and taking into consideration the known interactions between ROS and scavenging enzymes and the fact that UV radiation induces ROS formation, the effect of UV radiation on scavenging enzymes in the skin is not only to decrease or impair the enzyme activities as reported to date [6, 7, 10, 24, 36, 38], but also to induce their activities after the initial decrease. In addition, we hypothesize that tanning is, at least in part, due to UV radiation-induced ROS. Black also suggested the potential involvement of ROS in UV radiation-mediated cutaneous damage [3]. A direct correlation between ROS formation and the induction of melanogenesis by UVB radiation remains to be demonstrated.

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