Effects of Single-Dose Ultraviolet Radiation on Skin Superoxide Dismutase, Catalase, and Xanthine Oxidase in Hairless Mice

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The effects of a single exposure to UVB radiation on skin antioxidant enzymes and superoxide-generating xanthine oxidase were examined in Skh:HR-1 hairless mice. Significant decreases in superoxide dismutase (SOD) and catalase (CAT) were observed by 12 h after UV irradiation and remained depressed for up to 72 h. No induction of xanthine dehydrogenase (XD) or xanthine oxidase (XO) occurred with UV treatment, although significant hyperplasia was evident. Ornithine decarboxylase was induced after UV irradiation as has been previously reported. These results demonstrate significant biochemical effects of a single dose of UVB on murine epidermis, especially in terms of antioxidant defenses. *J Invest Dermatol 95:213–216, 1990*

The importance of free radicals in radiation carcinogenesis has been known for some time [1,2]. Their role in chemical carcinogenesis has also been studied extensively in recent years [3,4]. The objective of the present work was to examine the role of the enzyme xanthine oxidase and its generation of the superoxide anion, in epidermal carcinogenesis induced by ultraviolet B radiation (UVB). It has previously been reported that treatment with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) induces synthesis of the purine salvage pathway enzyme xanthine dehydrogenase (XD) and converts it to the superoxide-generating form xanthine oxidase (XO) [5,6]. The present study examined the potential for a single tumor promoting dose of UVB to induce these enzymes.

Other parameters examined included the activities of ornithine decarboxylase (ODC), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-PX). Since these enzymes diminish with TPA treatment, they appear to play a role in TPA tumor promotion [7,8]. We attempted to compare the behavior of these enzymes following UVB treatment with their reported behavior following TPA treatment. The induction of ODC by UVB has been previously reported by Verma et al [9] and was used as a positive marker for UVB exposure in these studies.

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Abbreviations:
CAT: catalase
GSH-PX: glutathione peroxidase
ODC: ornithine decarboxylase
SOD: superoxide dismutase
TPA: 12-O-tetradecanoylphorbol-13-acetate
UVB: ultraviolet B
XD: xanthine dehydrogenase
XO: xanthine oxidase

MATERIALS AND METHODS

Animals Female hairless mice of the Skh:HR-1 strain were obtained from Temple University and used for experimentation at 6–8 weeks of age.

Exposure to UVB A single dose of UVB (290–320 nm) from a Westinghouse FS-40 sunlamp was used so that the total energy exposure of the mice was 0.09 J/cm^2. UV radiation was administered by placing cages containing a maximum of five mice at a fixed distance (30 cm) from the light source. The total energy output of the lamps at skin surface was measured immediately before each experiment using a calibrated UVB LM HO6 C meter (National Biological Corp.) (0.70 ± 0.02 mW/cm^2 at a distance of 30 cm). The energy exposure of the mice was then calculated at 0.09 J/cm^2 and irradiation time was approximately 130 ± 4 sec. This was a dosage used previously to induce ODC [9] and has been determined to be 3 times the minimal erythematous dose for Caucasian volunteers [9].

Epidermal Preparations Mice were killed by cervical dislocation at 6, 12, 24, 48, and 72 h following the single dose of UVB. The dorsal skin was excised and the epidermis scraped with a razor blade, as described previously [5], and homogenized in 50 mM $NaKPO_4$, 0.1 mM EDTA, pH 7.0, and centrifuged at 13,000 × *g*. The supernatant fluid was immediately used for (XD + XO)/XO assays on the same day without freezing. Additional aliquots were stored at −70°C until used in the SOD, CAT, or GSH-PX assays. Skin homogenates used for the determination of ODC activities were made in the same buffer containing 0.2 mM pyridoxal phosphate and 5.0 mM dithiothreitol and assayed on the same day. Skin samples were also examined histologically to determine the extent of hyperplasia with UVB treatment. Full thickness strips of skin were placed in 10% buffered formalin for routine histologic processing and stained with hematoxylin and eosin for microscopic examination.

Ornithine Decarboxylase Activity ODC activity was determined in the soluble 13,000 × *g* supernatant by measuring the release of $^{14}CO_2$ from L-[^{14}C] ornithine (ICN Biomedicals Inc., spec. act. 50–60 mCi/mmol) according to the method of Weeks and Slaga [10]. The assay mixture contained 50 mM sodium potassium

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phosphate (pH 7.0), 0.2 mM pyridoxal phosphate, 5.0 mM dithiothreitol, 0.1 mM EDTA, 10 mM L-ornithine containing 0.25 μCi of L-[^{14}C] ornithine, and 300 μl of epidermal extract in a final volume of 1.0 ml. Plastic centrifuge tubes were equipped with rubber stoppers and center well assemblies (Kontes), where CO_2 was absorbed by 0.2 ml ethanolamine in the wells. After incubation at 37° for 60 min the reaction was stopped by the addition of 0.5 ml of 1 M citric acid. Reaction tubes were left to incubate for an additional 1 h at room temperature to assure complete absorption of $^{14}CO_2$ by the ethanolamine. Finally, the center well containing the ethanolamine was transferred to a 20 ml vial containing 7 ml of toluene-based scintillation cocktail (Beckman Ready Value) and 3 ml methanol. Radioactivity was measured in a Beckman LS-3801 scintillation counter. Specific activity was calculated as nmol CO_2 produced/h/mg protein. Protein content for all enzyme assays was determined with the Bio-Rad protein assay reagent using bovine serum albumin as a standard. Assays were always performed in duplicate, and blanks contained only buffer.

Catalase Activity CAT activity was determined by following the decomposition of hydrogen peroxide, measured as a decrease in absorbance at 240 nm, according to Aebi [11], using a Beckman DU-6 UV-Vis spectrophotometer. CAT activity as determined by this method reflects both the catalytic and peroxidic functions of the enzyme in the removal of hydrogen peroxide in biologic material and is the method of choice for assay of tissue extracts [11]. Because both properties of the enzyme function in vivo, it is appropriate to measure total catalase/peroxidase activity as CAT for the purposes of defining antioxidant defenses. Although this method could ostensibly allow detection of the degradation of H_2O_2 by other enzymes present in crude tissue extracts, the catalase/peroxidase activity demonstrated in our results using this method was totally inhibited by the presence of 1 mM NaN_3, a known inhibitor of catalase [12]. The initial concentration of hydrogen peroxide in the assay mixture was 10 mM. A unit of activity was calculated by using $k = (2.303/t)(\log A_1/A_2)$, where k is the rate constant, t is time in minutes, A_1 is absorbance at time O, and A_2 is absorbance at 2–4 min. Specific activity was calculated as k/mg of protein.

Superoxide Dismutase Activity SOD activity was determined in epidermal extracts according to the reduction of ferricytochrome c method of McCord and Fridovich [13]. The SOD assay mixture contained 50 mM phosphate buffer, 100 μM ferricytochrome c (Type III, Sigma), 50 μM xanthine, and 5 nM xanthine oxidase in a reaction volume of 1 ml. The reaction was started by the addition of 50 μl of epidermal extract and the absorbance followed at 550 nm for 2–4 min. One unit was defined as the amount of SOD sufficient to inhibit the rate of reduction of cytochrome c by 50% and specific activity expressed as units/mg protein.

Glutathione Peroxidase Activity GSH-PX activity was assayed by the coupled reaction with glutathione reductase according to the method of Paglia and Valentine [12]. A reaction of 1 ml contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 1 mM oxidized glutathione, 0.2 mM NADPH, and 1 unit/ml glutathione reductase. The reaction was monitored at 340 nm, and an extinction coefficient of 6.22 M^{-1} cm^{-1} was used to calculate units of activity.

Xanthine Dehydrogenase/Oxidase Epidermal extracts were assayed for the activities of both XD and XO by a modification of the procedure of Mousson et al [14]. A detailed description has been published [5]. Total XD + XO activity was distinguished from XO activity by including NAD+ in the incubation mixture. Specific activity is expressed as nmoles of hypoxanthine oxidized to xanthine plus uric acid/min/mg protein.

Statistical Analysis Statistical analyses were performed using Student t test or least significant differences where appropriate. They were calculated according to the methods of Sokal and Rohlf [15]. All data presented are the means ± SEM of three mice per treatment group combined from three separate experiments.

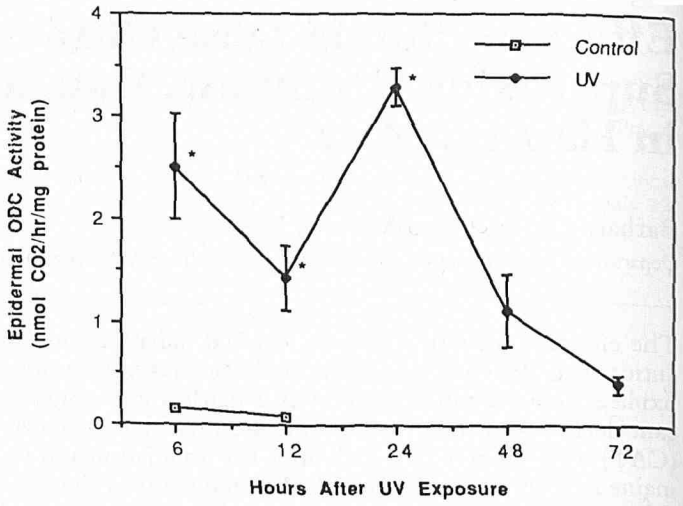

Figure 1. The effects of UVB irradiation on murine epidermal ODC activity at 6, 12, 24, 48, and 72 h after exposure to UVB (0 or 0.09 J/cm²). Points represent the mean ± SEM of three experiments using three mice each. The *asterisk* indicates significant ($p < 0.05$) difference from control values.

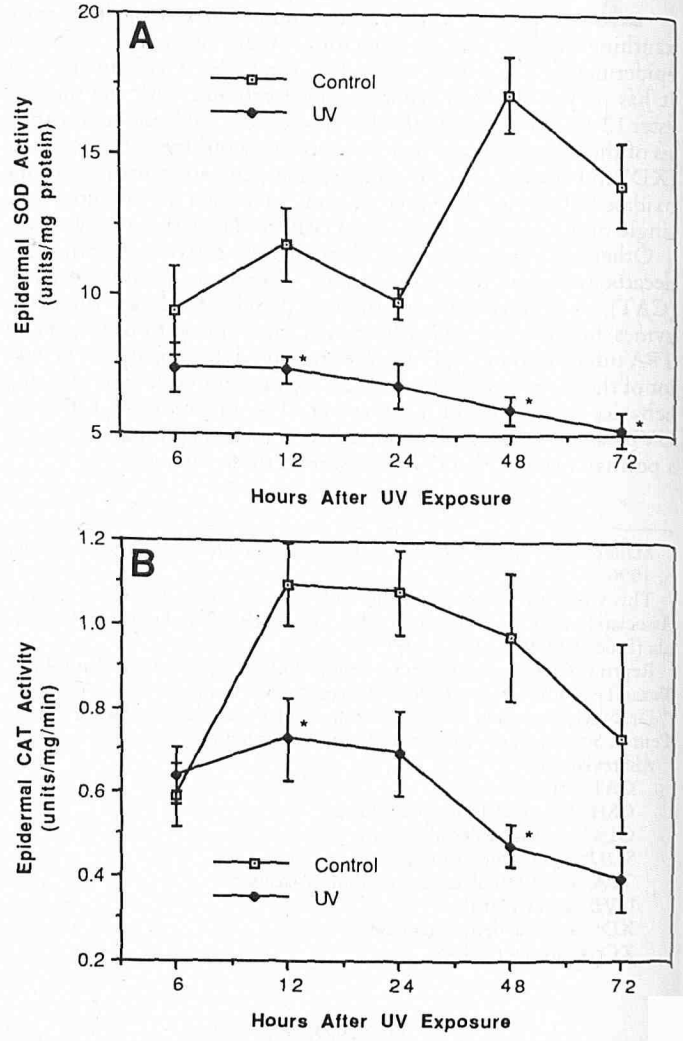

Figure 2. The effects of UVB irradiation on murine epidermal SOD *(panel A)* and CAT *(panel B)* activities at 6, 12, 24, 48, or 72 h after exposure to UVB (0 or 0.09 J/cm²). *Points* represent the mean ± SEM of three experiments using three mice each. The *asterisk* indicates significant ($p < 0.05$) difference from control values.

RESULTS

As shown in Fig 1, ODC was induced 17 times by 6-h post-UVB (as previously reported), dipped at 12 h, peaked in activity at 24 h, and then gradually declined to normal values. This is in agreement with the previous findings of Verma et al [9].

SOD and CAT activities compared to control values were decreased following UVB treatment and continued to stay depressed up to 72 h post-UVB as shown in Fig 2*A,B*. Miyachi et al [16] have also reported decreased skin SOD activity following a single exposure to UVB radiation, but the dose used in that study was 1.0 J/cm², more than 10-fold higher than that used in the present study. Interestingly, control values for both enzymes were elevated at the 12-h point, perhaps because of a circadian variation in these parameters during the nighttime hours when the mice are usually the most active. Additionally, there was an unexplained elevation of both SOD and CAT in control groups at 48 and 72 h, perhaps because of slight induction of these enzymes during manipulation of the animals. This phenomenon was also seen by Solanki et al [7]. The apparently elevated SEM for CAT seem to be comparable to those reported in other studies [7,17,18] of CAT measured in crude epidermal homogenates from SENCAR mice.

There was none to minimal elevation of XD/XO at any time following UVB treatment (Fig 3). This is unlike the findings previously reported after treatment with TPA [5,6]. Although hyperplasia is evident at 48 h following UVB treatment (Fig 4) there is no accompanying induction of either total XD + XO activity or increased conversion of existing XD to XO, as demonstrated with TPA treatment [5,6]. In the previous study [5], TPA induction of XD/XO peaked at 48 h, as did the hyperplasia.

Although GSH-PX activity did not give a consistent response across experiments, no significant differences were attributable to UVB treatment. Table I shows that the reported decrease in GSH-PX following TPA treatment of Sencar epidermal cells [8] is not evident after UVB exposure.

DISCUSSION

A single exposure of SKH:hr-1 hairless mice to UVB radiation resulted in numerous biochemical and histologic changes in the epidermis. The hypothesis we sought to test in this series of experiments was that UVB treatment may involve similar (to that of TPA treatment) mechanisms of epidermal damage via the generation of reactive oxygen species. As is demonstrated by the data showing decreases in SOD and CAT activities following a single UV treat-

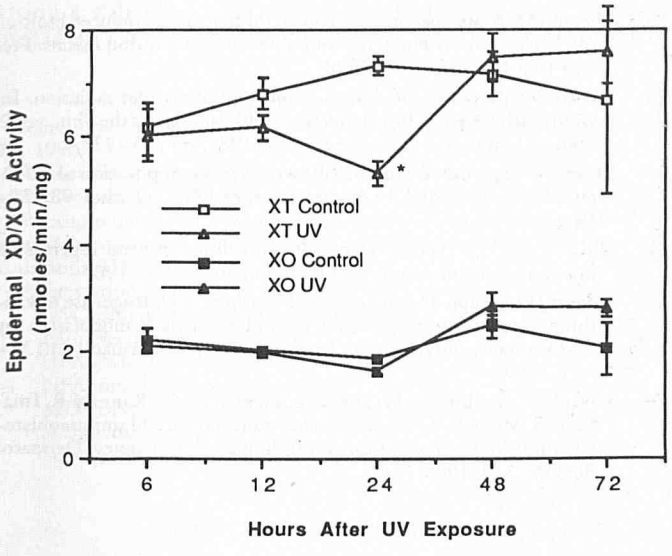

Figure 3. The effects of UVB irradiation on murine epidermal XO and XT (total XO + XD) activities at 6, 12, 24, 48, and 72 h after exposure to UVB (0 or 0.09 J/cm²). *Points* represent the mean ± SEM of three experiments using three mice each. The *asterisk* indicates significant ($p < 0.05$) difference from control values.

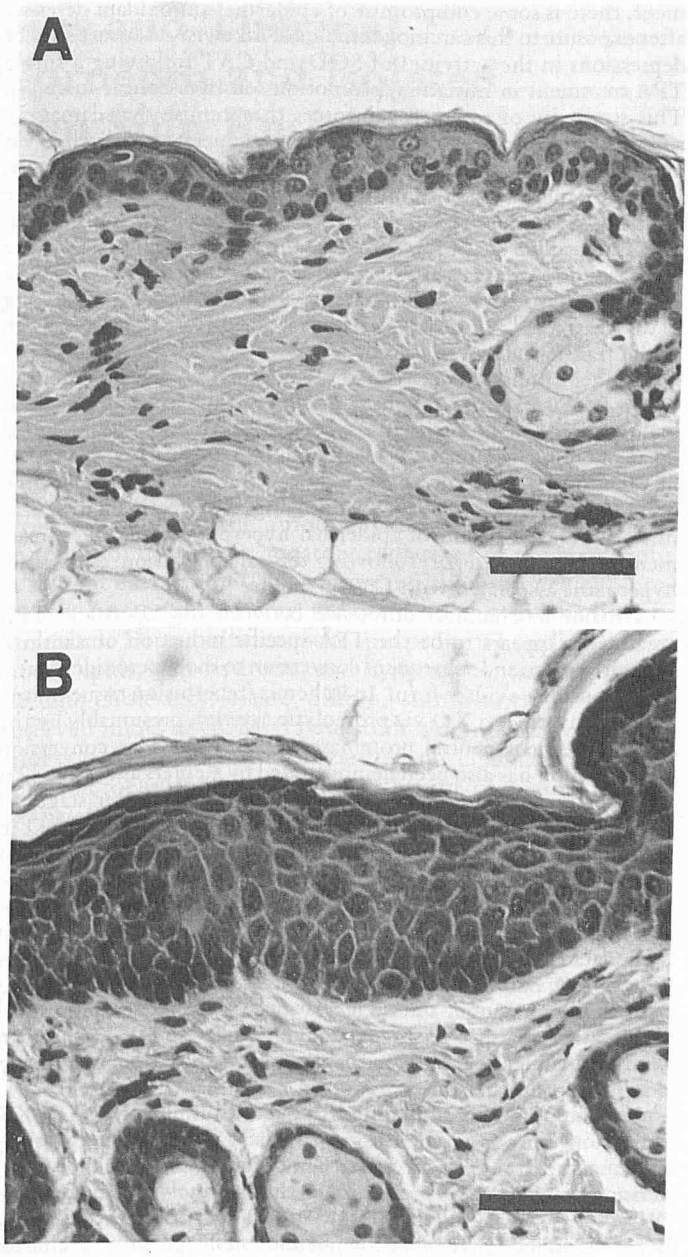

Figure 4. The effects of UVB irradiation on murine epidermal hyperplasia 48 h following sham *(panel A)* or UVB *(panel B)* treatment (0.09 J/cm²). *Scale bar:* 50 μm.

Table I. Epidermal Glutathione Peroxidase Activity[a] Following UVB Exposure (% of Control)

	Experiments		
Hours after UV	I	II	$\bar{X}$[b]
6	93.6	94.5	94.1
12	90.2	77.9	84.1
24	—	97.1	—
48	—	127.3	—
72	—	72.9	—

[a]GSH-PX activity (units/mg protein) in murine epidermal homogenates.
[b]Mean of two experiments.

ment, there is some compromise of epidermal antioxidant defenses after exposure to this carcinogenic agent. Likewise, there are similar depressions in the activities of SOD and CAT following a single TPA treatment in initiation/promotion sensitive Sencar mice [7]. This similarity of response reinforces the premise that damaging reactive oxygen species are thought to be generated by both UVB and TPA. In the case of UVB, we speculate that this damage results in either i) increased consumption of the enzymes in response to the oxidant stress induced by UVB or ii) UVB-induced enzyme destruction at the active Cu or Fe centers via the Fenton reaction [19]. The decreases do not appear to be a result of a UVB-induced delay in DNA synthesis because the kinetics of DNA synthesis following UVB treatment [20] shows increased rates at 48 to 72 h following an initial transient delay at 1–6 h.

Another similarity between the epidermal changes elicited by UVB and TPA is the resultant induction of ODC and the development of epidermal hyperplasia, with similar kinetics occurring for these phenomena after both UVB and TPA treatments. Induction of ODC in hairless mice by UVB occurs at about the same time point (6 h) as induction of ODC following TPA treatment in Sencar mice [9]. Development of epidermal hyperplasia after TPA treatment peaks at 2–3 d [21] following topical application, as does the hyperplasia associated with UVB exposure [9].

A major biochemical difference between the UVB and TPA treatments appears to be the TPA-specific induction of xanthine dehydrogenase and subsequent conversion to the superoxide-generating xanthine oxidase form. In ischemia/reperfusion tissue injury, XD is converted to XO via proteolytic activity, presumably by the Ca^{++}-dependent neutral proteinase calpain [22]. This conversion phenomenon has also been demonstrated by Reiners and Rupp [23] to occur in epidermis, predominantly during the latter stages of keratinocyte differentiation. Their study suggests that the XD to XO conversion occurs primarily in the squamous layer, and that conversion is mediated by proteases as well. Miyachi et al [24] have reported that calpain I in human skin was localized in the mid-to-upper epidermis but not in the basal cells. This finding is consistent with data from studies of TPA-induced XD synthesis and conversion to XO, but apparently calpain activation, the XD to XO conversion phenomenon, and their relationship to epidermal terminal differentiation are TPA-specific and not related to UVB treatment. The involvement of epidermal calpain in chemical carcinogenesis is a subject for further investigation.

Previously published findings [6] have also linked this XD/XO induction/conversion to the hyperplasia accompanying various treatments with phorbol esters. Here we demonstrate the occurrence of epidermal hyperplasia with absolutely no increase in XD/XO, as is seen with TPA [5,6]. Thus, it appears that the XD/XO induction/conversion phenomenon is possibly specific for TPA-dependent and other chemical promoter-dependent hyperplasias and not for that induced by any other source. Additionally, perhaps UVB treatment does not result in accelerated terminal differentiation, as does TPA, but in merely a regenerative hyperplasia in response to generalized cellular damage. Many questions remain to be answered concerning the role of oxygen radicals, antioxidants, and proteolysis in the development of epidermal carcinogenesis.

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