DISTRIBUTION OF CHOLESTEROL-5α,6α-EPOXIDE FORMATION AND 
ITS METABOLISM IN MOUSE SKIN

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The distribution of cholesterol-5α,6α-epoxide in ultraviolet light-irradiated mouse skin was determined. Highest levels of cholesterol-5α,6α-epoxide were found in the epidermis and distributed rather evenly throughout all subcellular fractions. Low but potentially significant levels of the compound diffused from the epidermis. Metabolic studies demonstrated that cholesterol-5α,6α-epoxide was converted to cholestan-3β,5α,6α-triol by an enzyme, cholesterol-5α,6α-epoxide hydrase, which was localized in the epidermal fractions sedimenting at 10,000 × g. Comparative studies indicated a much higher capacity for metabolism of cholesterol-5α,6α-epoxide in liver than in skin. The effect of the epoxide and its metabolic product on the skin is discussed.

Numerous arene oxides have been shown to be carcinogenic or mutagenic. Some of these are even more potent than their parent hydrocarbons [1-4]. Several alkene oxides have also been reported to be effective carcinogens or mutagens [5-7]. It has recently been suggested that a sterol oxide might be involved in the etiology of ultraviolet light (UVL)-induced carcinogenesis [8]. For the most part, sterol oxides have been thought to be rather limited in their natural distribution. However, a particular sterol, cholesterol-5α,6α-epoxide (CAE), has been reported to be present in serum from patients with elevated cholesterol levels and has been found as a constituent of cholesterol-derived photoproducts in both human skin and that of hairless mice after exposure to UVL [9-12]. It is known to possess carcinogenic properties [13].

In the case of hydrocarbon-derived arene oxides, an enzyme, epoxide hydrase, converts such oxides into far less reactive dihydrodiols [14,15]. The importance of arene oxide metabolism lies in (1) the detoxification of carcinogenic compounds to less harmful ones, (2) the possible alteration of this detoxification potential by environmental factors thus leading to the malignant state, and (3) the potential manipulation of this metabolism as a therapeutic tool.

Consequently, the metabolism of CAE may be of critical importance in the developmental sequence of UVL-induced carcinogenesis. The studies reported here provide information on the distribution and reaction rates of CAE hydrase as well as the distribution of UVL-induced CAE formed in mouse skin.

MATERIALS AND METHODS

Localization of Cholesterol-5α,6α-Epoxide

Four female albino hairless mice were used for each study. The animals were decapitated and dorsal skin from the shoulder to hip and extending ventrally to the midquadrant was removed. The skin was trimmed and subcutaneous tissue scraped away. The intact sheets of skin were incubated for 24 hr with constant shaking at 4°C in a mixture of 0.5 ml Tween 80 solution (86.6 mg/100 ml 1% ethanol; 4.0 ml Krebs-Ringer phosphate buffer, pH 7.4; and 2 μCi of [4-14C]cholesterol (specific activity, 60 mCi/mm)).

After the incubation period the tissue was removed and thoroughly rinsed with Tween 80 solution and deionized water to remove surface contamination by labeled cholesterol. The tissue was blotted dry between sheets of filter paper and divided into two groups. One group was irradiated for 30 min with a Burdick QA-150N mercury arc lamp (1.43 × 10^4 ergs/cm^2/sec) in an environmental chamber maintained at 37°C. Controls were maintained under the same experimental conditions except for irradiation.

Following irradiation both groups were trypsinized for 45 min at 40°C as described by Fan [16] (except that 0.6% trypsin solution (12,500 BAEE units/mg protein; Sigma Chemical Co.) was employed. Epidermis was separated from dermis after the trypsin treatment, both were thoroughly rinsed, minced, and homogenized in 4 ml of deionized water with a Model 10 Polytron homogenizer equipped with a saw-toothed generator. The total lipids were extracted 4 times with chloroform:methanol (2:1, v/v), dried under nitrogen, streaked on thin-layer chromatography (TLC) plates (SilicaAR-7GF, 20 × 20 cm, 250 μm), and co-chromatographed with nonlabeled authentic cholesterol and CAE in 1.2-dichloroethane.

For subcellular localization of 14C-labeled polar materials, the preceding procedure was followed except that only epidermis was used and this tissue was homogenized in 9.0 ml of Krebs-Ringer phosphate buffer solution. The homogenate, containing 3.5 mg protein/ml, was centrifuged at 500 × g for 10 min to remove cell debris and...
whole cells. The resulting supernatant was then centrifuged at 2,000 × g for 10 min. After removal of the pellet, the supernatant was centrifuged at 10,000 × g for 30 min. The resulting pellet was retained and the supernatant centrifuged at 100,000 × g for 60 min. The pellet obtained after each centrifugation was washed with buffer solution, centrifuged, and the resulting supernatant combined for the next centrifugation. Each fraction was subjected to lipid extraction and TLC separation as described.

**Diffusion of Cholesterol-5α,6α-Epoxide**

Epidermis, obtained as previously described, was spread evenly across the surface of small Petri dishes containing 5 ml of 1.5% agar in Krebs–Ringer phosphate buffer solution. The epidermis obtained from one animal was irradiated with UVL for 30 min in the environmental chamber. The control epidermis was maintained under similar conditions except for irradiation. An additional control was maintained in which the agar was preirradiated prior to addition of the epidermis. After the irradiation period the dishes were incubated in the dark at room temperature for 24 hr. At the end of the incubation period, epidermis and agar were separated, homogenized, total lipids extracted, and 14C-labeled polar materials analyzed as described.

**Reaction Rate of Cholesterol-5α,6α-Epoxide Hydrase**

Fresh skin or liver was homogenized in a volume of buffer equivalent to 4 times the fresh weight. The homogenate was centrifuged for 10 min at 500 × g and the supernatant was used for enzyme studies. All procedures were performed at 4°C. The reaction mixture contained 9 nmole of 1,2-3Hcholesterol-5α,6α-epoxide (0.075 μCi, specific activity, 8.28 mCi/mmol), 50 μl of Tween 80 solution, and 1 ml of supernatant. Incubations were carried out at 37°C for 30 min in 10 × 100 mm test tubes. Incubations containing boiled enzyme and no enzyme (buffer solution) served as controls.

After the incubation period, the reactions were terminated by the addition of 5.0 ml of chloroform:methanol. Total lipids were extracted and co-chromatographed with nonlabeled cholesterol-5α,6α-epoxide and cholestane-3β,5α,6β-triol in chloroform:acetone (9:1, v/v). Bands corresponding to the Rf values of each of the cold standards were scraped off and radioactivity determined. A Packard Model 3375 liquid scintillation spectrometer was used for all radioactivity measurements. Mean counting efficiencies were 78% and 34% for carbon and tritium, respectively. The percent standard deviation of replicate counts was always 2.5 or less. Quenching was determined by the automatic external standardization method.

Enzyme activity was calculated as the percent of conversion of radioactive cholesterol-5α,6α-epoxide to cholestane-3β,5α,6β-triol per mg protein of homogenate. Incubations containing boiled enzyme or no enzyme, which served as controls, contained less than 0.1% polar material.

Radioactive CAE was prepared by slowly adding 120 μl (10.9 mg) of m-chloroperoxybenzoic acid (Research Organic/Inorganic Chemical Corp., Belleville, N. J.) in chloroform to a 15 × 100 mm Pyrex test tube containing 0.25 mCi of labeled cholesterol and 19 mg of cold cholesterol in 75 μl of chloroform. After thorough mixing for 1 min, 5 ml of chloroform was added. The reaction was completed in 20 min and the excess m-chloroperoxybenzoic acid was destroyed by slow addition of 10% sodium sulfite until a starch–iodide paper test was negative. The aqueous layer was removed and the organic layer washed 3 times with 10% sodium bicarbonate solution, and, finally, with saturated sodium chloride. The organic layer was dried and stripped of solvent. The residue was crystallized from aqueous acetone to give a 98% yield of CAE.

**Subcellular Localization of Epoxide Hydrase**

The epidermis from 4 animals was used in each study and treated as described above except that 250 ml sucrose was added to the buffer solution. Each pellet obtained from centrifugation was resuspended in 3 ml of buffer, and 1.0 ml was used for each of the duplicate incubations. The remaining 1.0 ml was used for protein determination [17]. Incubation, extraction, isolation, and calculation of enzyme activity of each fraction were the same as described for whole skin.

**RESULTS AND DISCUSSION**

When aqueous suspensions of [14C]cholesterol were exposed to broad-spectrum radiation from a mercury arc lamp, CAE was identified as one of the polar photoproducts formed [18]. Light of wavelengths 254 and 265 nm was most effective in alteration of cholesterol. Bischoff [12] has shown that CAE, when injected subcutaneously, will produce sarcomas in both rats and mice. As CAE was found as a constituent of cholesterol-derived photoproducts in human skin irradiated with UVL, it was suggested that this sterol might play some role in the etiology of UVL-induced carcinogenesis [11]. Subsequent studies, using the hairless mouse as a model system, revealed that upon chronic UVL irradiation, the CAE levels in skin increased 2- to 8-fold prior to the onset of UVL-induced tumors [8]. The resulting tumors were of epidermal origin. Blum and co-workers have demonstrated that 90% of the UVL is absorbed by the dorsal epidermis of the mouse ear [19,20]. It seemed reasonable, therefore, to determine the CAE levels in both dermis and epidermis and the relationship between the tissue of tumor origin and the primary UVL target tissue.

It can be seen in Table I that when whole skin is irradiated and the dermis and epidermis are subsequently separated, levels of polar cholesterol pho-

| TABLE I. Localization of [14C]-cholesterol-5α,6α-epoxide formation in mouse skin |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 1st Exp         | 2nd Exp         | 3rd Exp         | Average         |
| Irradiated epidermis | 371             | 256             | 590             | 406             |
| Irradiated dermis    | 116             | 86              | 150             | 122             |

[14C]-Polar material refers to the radioactive photoproducts, in the total lipid extract, that remain at the origin of TLC plates after development in 1,2-dichloroethane. The polar fraction contains CAE. Composition of this fraction has previously been described [9,11]. The levels of [14C]-polar materials found in irradiated tissues are expressed here as the percentage of those found in nonirradiated controls.
toproducts, of which CAE is a constituent [9], increase 4-fold in the epidermis. The percent increase of these materials in the dermis is only slight (22%). In all experiments, almost equal amounts of labeled cholesterol are taken up by both epidermis and dermis, with slightly higher uptake levels occurring in the epidermis. The data in Table II indicated that formation of the CAE is distributed rather evenly among all cellular fraction of the epidermis. These data are consistent with the thesis of CAE involvement in UVL carcinogenesis in that CAE is preferentially formed in the epidermis—the tissue of origin for most UVL-induced tumors in the hairless mouse.

On the other hand, as seen in Table III, the polar photoproducts, of which CAE is a constituent, are capable of diffusing out of the epidermis. It is of interest to note that CAE is itself a vessel dilator (John Wolf, personal communication). Thus, diffusion of CAE to the dermis could allow this compound to participate in the events resulting in UVL-induced erythema.

![Table II. Subcellular localization of \(^{14}C\)cholesterol-5a,6a-epoxide in mouse skin epidermis](image)

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Protein (mg)</th>
<th>([^{14}C])cholesterol uptake (cpm)</th>
<th>([^{14}C])cholesterol uptake (cpm/100,000 proteins)</th>
<th>([%]^{14}C) Polar material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal homogenate</td>
<td>2.6</td>
<td>552</td>
<td>212</td>
<td>14.2</td>
</tr>
<tr>
<td>500 × g pellet</td>
<td>0.6</td>
<td>82</td>
<td>137</td>
<td>13.8</td>
</tr>
<tr>
<td>2,000 × g pellet</td>
<td>0.4</td>
<td>45</td>
<td>113</td>
<td>15.5</td>
</tr>
<tr>
<td>10,000 × g pellet</td>
<td>0.5</td>
<td>78</td>
<td>156</td>
<td>16.0</td>
</tr>
<tr>
<td>100,000 × g pellet</td>
<td>0.2</td>
<td>29</td>
<td>145</td>
<td>18.0</td>
</tr>
<tr>
<td>100,000 × g supernatant</td>
<td>1.6</td>
<td>14</td>
<td>9</td>
<td>18.5</td>
</tr>
</tbody>
</table>

![Table III. Diffusion of \(^{14}C\)-polar material from the epidermis](image)

Sheets of mouse epidermis were spread on an agar surface. After irradiation, total lipids were extracted from the agar and epidermis respectively. The percent of the total radioactivity found in the polar fraction from each treatment is reported. Controls were treated in similar fashion except for irradiation. There was no difference in epidermal CAE diffusion when nonirradiated or preirradiated agar was used.

<table>
<thead>
<tr>
<th>% (^{14}C)-Polar material</th>
<th>1st Expt</th>
<th>2nd Expt</th>
<th>3rd Expt</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar (nonirradiated epidermis)</td>
<td>5.3</td>
<td>7.0</td>
<td>10.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Agar (irradiated epidermis)</td>
<td>13.3</td>
<td>17.0</td>
<td>17.3</td>
<td>15.8</td>
</tr>
<tr>
<td>Epidermis (nonirradiated)</td>
<td>3.0</td>
<td>4.6</td>
<td>2.7</td>
<td>3.2</td>
</tr>
<tr>
<td>Epidermis (irradiated)</td>
<td>9.2</td>
<td>8.2</td>
<td>9.2</td>
<td>8.9</td>
</tr>
</tbody>
</table>

The distribution and localization of CAE could be of vital importance with respect to its accumulation and thus its carcinogenicity. It is obligatory that studies of this nature encompass the capacity of the host tissue to metabolize the compound under study. The Figure compares the conversion rate of [1,2,\(^3\)H]cholesterol-5a,6a-epoxide to cholesterol-3β,5α,6β-triol by cell-free homogenates of mouse skin and liver. Hydration of the CAE did not occur in control incubations of heat-treated preparations. Cholesterol-3β,5α,6β-triol was identified by relative retention time data from gas-liquid chromatography (GLC) after formation of a trimethyl silyl derivative, radio-gas chromatography, and co-crystallization to constant specific activity with the authentic sterol. As seen in the Figure, cholesterol-5α,6a-epoxide hydrazide activity was much greater in liver than in skin. The inefficient cutaneous metabolism of CAE when compared to liver (Fig.), could account for the greater sensitivity of skin to any physiologic response that might be evoked by this compound. Furthermore, the data in Table IV indicate that the preponderance of CAE hydrazide activity in skin occurs in the epidermis. This coincides with the data in Table I in which UVL-induced CAE formation occurred primarily in the epidermis and it suggests a substrate-inducible relationship. The higher level of CAE hydrazide activity present in the epidermis of mouse skin can be caused by the preferential formation of UVL-induced formation of CAE in this tissue. The presence of a CAE metabolizing enzyme will no doubt affect the amount of detectable CAE in a biologic sample at any given time. A previous study [21] indicated that when hairless mice were subjected to chronic UVL irradiation there was a significant increase of CAE hydrazide activity after 8 weeks of UVL irradiation. The increased activity reached a maximum at 15 weeks and remained at a substantially higher level than that of controls through 20 weeks. The initiation of higher activities of CAE hydrazide corresponded to an increased level of CAE. However, CAE reached the highest level prior to the increase in CAE hydrazide activity, indicating that the primary effect of UVL is on the formation of CAE which, in turn, affects the level of CAE hydrazide through an enzyme-substrate induction relationship.

As the highest enzyme activity is found in the epidermis, this tissue can be used to determine the enzyme’s subcellular distribution. Table V indicates that the highest CAE hydrazide activity is associated with the 10,000 and 100,000 × g pellets. This finding is in agreement with similar studies of epoxide hydrazide enzyme from other tissues. Oesch et al [22,23] reported epoxide hydrazide activity of human liver biopsy specimens to be predominantly associated with the 100,000 × g fraction. Stoming and Bresnick [24] found the major epoxide hydrazide activity in rat tissues was associated with the 100,000 × g fraction. The results in Table V differ from those reported for adult rat brain [25] in that...
Epidermal fractions which sediment at 600 g demonstrated that these particles are most active in cell-free amino acid incorporation. Thus, the epoxide hydrazide activity in 500, 2,000, and 10,000 g pellets reported in Table V could be attributed to incomplete fractionation of the epidermis.

Although CAE is known to possess carcinogenic properties, it is formed in UVL-exposed skin, and increased levels of the compound precede the onset of tumors in chronically UVL-exposed mice. No definitive evidence has yet linked CAE to a causal role in UVL-induced carcinogenesis. Nevertheless, the formation of this compound in skin represents a response of this tissue to both acute and chronic exposure to UVL. Other studies have demonstrated the toxicity of CAE to specific tissues and attributed this toxicity to the compound's conversion to cholestane-3β,5α,6δ-triol. The latter is known to inhibit cholesterol biosynthesis [27].

The fact that the formation of CAE results from an acute or chronic exposure to UVL and that cholestane-3β,5α,6δ-triol results as a metabolic product of CAE, suggests that either or both participate in the myriad of physiologic responses to UVL insult. This paper has dealt with the distribution of CAE formation, its metabolism, and the distribution of an enzyme responsible for that metabolism. The importance of CAE or its metabolic products to the total physiology of the epidermis must await further investigation.

We thank Mr. Jack O. Ford for technical assistance.

REFERENCES


5. Van Duuren BL, Langseth L, Lorriss L, Baden M, Kushner M: Carcinogenicity of epoxides, lactones, and very little CAE hydrazide activity was found in the 105,000 × g supernatant.

Freedberg [26] has found small but significant amounts of ribosomes in homogenized guinea-pig epidermal fractions which sediment at 600 × g. He demonstrated that these particles are most active in cell-free amino acid incorporation. Thus, the epoxide hydrazide activity in 500, 2,000, and 10,000 × g pellets reported in Table V could be attributed to incomplete fractionation of the epidermis.