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Anti-inflammatory Properties of an Oxidized Sterol

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A polar photoproduct of cholesterol oxidation, 7-ketocholesterol, was able to inhibit in a dose-dependent manner the mouse ear-swelling response to irritants such as croton oil or cantharidin. Its anti-inflammatory properties were much less than equivalent concentrations of hydrocortisone, but the oxidized sterol did not induce any systemic effects (as measured by thymolytic activity), as did topical hydrocortisone. It is concluded that 7-ketocholesterol has weak anti-inflammatory activity, and its mode of action may be different from that of glucocorticoids.

Cholesterol can be oxidatively degraded by a thermochemical or photochemical reaction to form polar derivatives (e.g., oxidized sterols; see Fig 1) [1]. Lo and Black [2] have demonstrated that cholesterol oxidation is also a photobiologic event, as they have isolated these oxidized derivatives of cholesterol from human skin after ultraviolet irradiation. Black has suggested that this family of oxidized sterols may have great significance in the genesis of pathologic effects of ultraviolet irradiation upon human skin [3].

Many cell-culture studies with these polar sterols suggest that they may have glucocorticoid-like activity. They have been noted to be immunosuppressive [4], antimitotic and catabolic [5], antichemotactic [6], and to inhibit cellular intermediate metabolism [5,7]. Additionally, it has been demonstrated that mammalian cells possess internalized cytoplasmic receptors for oxidized sterols [8], as has been noted for glucocorticoids. Despite this abundance of promising in vitro data concerning the biologic activity of these compounds, there are few published studies that evaluate the possible in vivo glucocorticoid-like activity of this group of chemicals. It is, therefore, the purpose of this study to examine oxidized sterols for a well-characterized property of glucocorticoids, i.e., antiphlogistic activity. This was done using the ear-swelling assay developed by Swingle et al [9].

MATERIALS AND METHODS

Ear-Swelling Assay

Female Swiss-Webster mice (Jackson Laboratories, Bar Harbor, Maine), 6–8 weeks old and weighing 20–30 g, were fed standard mouse pellets and water ad lib. Each mouse's ear was irritated with 10 μl of croton oil or cantharidin (Sigma Chemical Co., St. Louis, Missouri) in acetone at a concentration of 2.5 mg/ml. These irritants were applied to the inner and outer surfaces of the right ear at time zero using an Eppendorf pipette. The left ear remained untreated. (Acetone alone did not induce any changes in ear weight.)

For croton oil-irritated ears, the peak ear swelling occurs at 6 h; for cantharidin-irritated ears, the swelling response maximizes at 8 h [9]. Ear swelling was measured at its respective peak for each irritant. This was done by obtaining a 4-mm punch biopsy from the inflamed and control ears of ether-anesthetized mice and immediately weighing them to the nearest 0.01 mg on a Mettler balance (Princeton, New Jersey). The difference in weight between the inflamed and control ears (ΔW) was calculated.

Topical treatments were applied 30 min after the irritants so as to minimize the possibility of any nonspecific physicochemical interaction between the irritant and the anti-inflammatory agent, which could result in a nonpharmacologic inhibition of the ear-swelling response.

These treatments were applied to the inner and outer surfaces of the inflamed ear. Crystalline hydrocortisone (Sigma Chemical Co.) and 7-ketocholesterol (Sigma Chemical Co.) were made soluble in a 4:1 acetone:ethanol solution, while cholesterol (Sigma Chemical Co.) was dissolved in acetone:ether:ethanol, 2:2:1.

Results are recorded as percent inhibition of the ear-swelling response, which was calculated using the following formula:

$$\% \text{ suppression of ear swelling} = \frac{\Delta W_C - \Delta W_T}{\Delta W_c}$$

ΔW_C = the difference in ear weights between the vehicle-treated inflamed ear and the untreated (control) ear. ΔW_T = the difference in ear weights between the compound-treated inflamed ear and the untreated (control) ear. Each treatment group consisted of 7 animals. Data are reported as arithmetic means ± SD in both tables and figures.

Measurement of Systemic Effects

Tonelli et al [10] have demonstrated that topically applied glucocorticoids induce premature involution of the thymus gland in rodents. Thus 48 h after topical application of the materials, the animals were sacrificed, and their thymus glands were removed and weighed and expressed as mg of thymus/100 g body weight.

Histologic Specimens

The 4-mm punch biopsies (obtained for weight measurements at the peak of the ear-swelling response) were fixed in neutral buffered formalin, paraffin embedded, and sectioned at 10 μm. Slides were stained with hematoxylin-eosin. Specimens from 5 mice in each group were examined.

Statistics

To test for the significance of the effects between the control groups and any individual treatment, a one-tailed *t*-test was used [11]; p values < 0.05 were considered significant.

RESULTS

Topically applied cholesterol did not interfere with croton oil-induced ear swelling at its lowest dose (2.5 μg), but there was suppression of the ear-swelling response at the 2 higher doses (Fig 2). (Only the 25 μg topical dose was statistically significant: $p < 0.05$).

Topical 7-ketocholesterol inhibited the ear-swelling response at all 3 doses studied and did so in a dose-dependent manner (statistical significance $p < 0.05$ at all doses studied). Locally applied hydrocortisone demonstrated a similar, somewhat more potent, dose-dependent inhibition of the ear-swelling response.

For cantharidin-induced ear swelling (Fig 3), cholesterol had no inhibitory effects on this type of irritant. Topical 7-ketocholesterol, again, inhibited this response in a dose-dependent manner, and statistical significance occurred only at the 25 μg and 250 μg topical doses ($p < 0.05$). Again, hydrocortisone was the most potent antiphlogistic agent, suppressing the ear-swelling response by 35%, 44%, and 65% with increasing topical doses.

To quantitatively assess the antiphlogistic effects of the

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Abbreviations:
ID 50: dose that inhibited ear swelling by 50%
PML: polymorphonuclear leukocytes

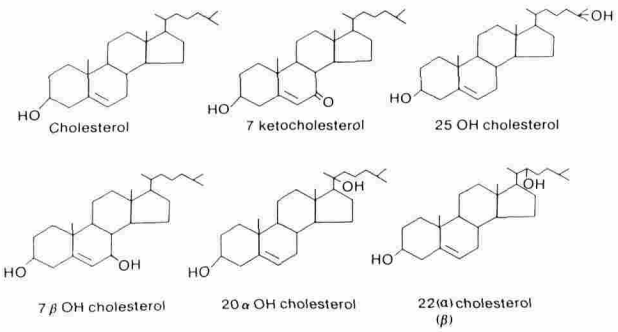

FIG 1. Cholesterol and its oxidized derivatives. Cholesterol oxidation products differ from parent compound by the introduction of a functional ketone or hydroxyl group in the 7, 20, 22, or 25 positions in the cholesterol nucleus.

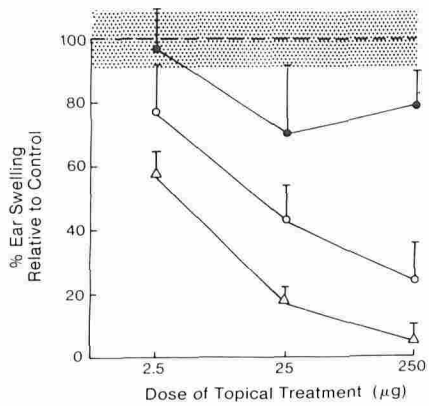

FIG 2. Inhibition of croton oil ear-swelling response by 7-ketocholesterol (○——○), cholesterol (●——●), hydrocortisone (△——△), or vehicle (----). Data points represent arithmetic mean of 7 mice; error bars represent SD.

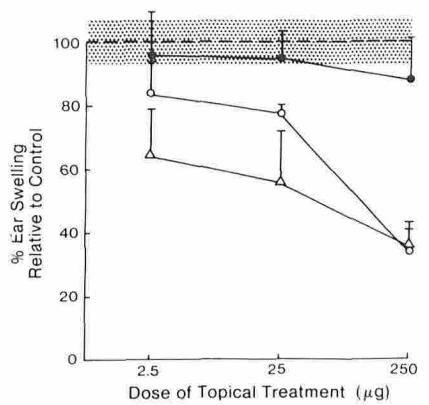

FIG 3. Inhibition of cantharidin ear-swelling response by 7-ketocholesterol (○——○), cholesterol (●——●), hydrocortisone (△——△), or vehicle (----). Data points represent arithmetic mean of 7 mice; error bars represent SD.

topical treatments that inhibited ear swelling, the dose that inhibited ear swelling by 50% (ID 50) was determined (Table I). It is apparent that hydrocortisone is more potent than 7-ketocholesterol against both types of irritants. Both 7-ketocholesterol and hydrocortisone were less effective agents against cantharidin irritation compared with croton oil irritation.

Mice treated with topical applications of cholesterol or 7-ketocholesterol did not demonstrate any significant thymic involution when compared with a vehicle-treated control group (Table II). Only topically applied hydrocortisone induced involution of the thymus gland, which was statistically significant at all topical doses studied. It is interesting to note that there is no suggestion of a dose-dependent induction of thymic involution, as all 3 doses induced approximately the same mean thymus/body-weight ratio.

Biopsies from irritated skin were examined to give further information about the anti-inflammatory properties of the oxidized sterol.

Croton oil-irritated skin demonstrated minimal epidermal irritation; the dermis was distended with edema fluid. There was a dense inflammatory-cell infiltrate scattered throughout the dermis consisting of mononuclear cells and polymorphonuclear leukocytes (PML). There was marked vasodilatation and a few extravasated red blood cells (Fig 4).

The skin that was inflamed and subsequently treated with 7-ketocholesterol demonstrated the same pathologic changes as the untreated skin, but these changes were less exaggerated. There was less dermal edema, vasodilatation was still present, and the mixed inflammatory infiltrate was reduced in density (Fig 5).

Cantharidin-irritated skin exhibited essentially the same dermal changes as did croton oil-inflamed ears, except that the degree of edema was greater. There was also marked epidermal change: keratinocyte necrosis, focal ulceration, vesiculation, and inflammatory-cell epidermal exocytosis were present (Fig 6). Again, treatment of this type of inflammation reduced the degree but not the character of the pathologic change (Fig 7). The epidermal pathology was not substantially altered by 7-ketocholesterol treatment.

DISCUSSION

This biologic assay demonstrates that 7-ketocholesterol, an oxidized derivative of cholesterol, has the ability to interfere with inflammatory (irritant) responses in a dose-dependent manner, suggestive of a pharmacologic response. The parent compound cholesterol lacked such activity. It is also apparent that this compound has less antiphlogistic activity than hydrocortisone, a glucocorticoid that is widely used in clinical and investigative settings and is a low-potency anti-inflammatory agent when compared with other glucocorticoids [10]. This

TABLE I. *Inhibition of croton oil- and cantharidin-induced ear swelling by topical agents*

	ID[a]	
	Croton oil	Cantharidin
Hydrocortisone	2.37 μg (1.19–5.62) = 0.09 mg/kg[b]	50.12 μg (28.18–84.14) = 2.00 mg/kg
7-Ketocholesterol	14.13 μg (6.68–28.18) = 0.57 mg/kg	141.25 μg (66.83–281.84) = 5.65 mg/kg

[a] Dose producing 50% inhibition of the increase in ear weight of the inflamed ear. ID 50 values calculated from the equation for a straight line determined from the regression equation of % inhibition vs log dose.

[b] Calculated for a 25-g mouse.

TABLE II. *Ratio of thymus weight (mg) to body weight (100 gr) of mice treated with topical agents*

	250 μg	25 μg	2.5 μg	0 μg
Vehicle	—	—	—	240.57 ±19.85
Cholesterol	213.19 ±73.70	216.34 ±33.71	214.42 ±31.92	—
7-Ketocholesterol	238.47 ±42.46	221.95 ±40.88	218.72 ±41.56	—
Hydrocortisone	177.34[a] ±20.77	185.16[a] ±33.24	186.81[a] ±27.73	—

[a] Statistically significant $p < 0.05$.

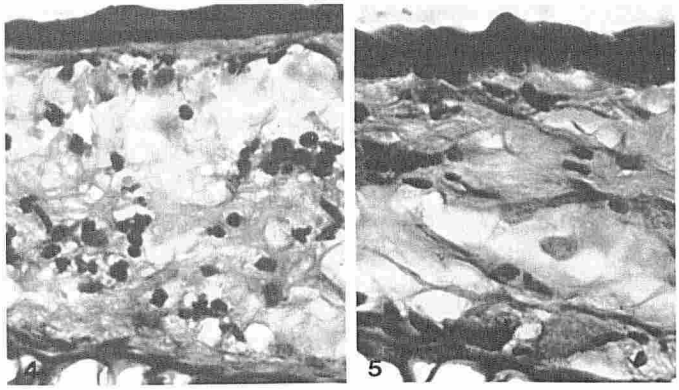

FIG 4. Light microscopy of croton oil-irritated mouse skin. There is little epidermal effect; the dermis is edematous, and there is vasodilatation with a mononuclear and polymorphonuclear infiltrate. Hematoxylin & eosin, × 40.

FIG 5. Light microscopy of croton oil-irritated skin treated with 7-ketocholesterol (250 μg). The dermal edema is reduced, and the density of the inflammatory infiltrate is decreased. Hematoxylin & eosin, × 40.

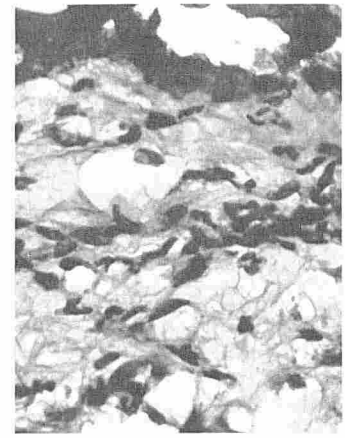

FIG 7. Light microscopy of cantharidin-irritated skin treated with 7-ketocholesterol (250 μg). Epidermal disruption is still present. Dermal edema is greatly reduced, as is the density of the inflammatory infiltrate. Hematoxylin & eosin, × 40.

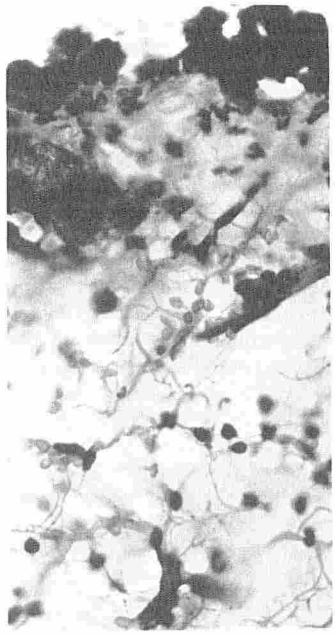

FIG 6. Light microscopy of cantharidin-irritated skin. The epidermis is disrupted; keratinocyte necrosis and epidermal vesiculation are present. The dermis is distended with edema fluid. A mononuclear and polymorphonuclear infiltrate is present along with extravasated erythrocytes. There is also exocytosis of inflammatory cells. Hematoxylin & eosin, × 40.

suggests that 7-ketocholesterol has weak topical potency relative to existing glucocorticoids.

It is interesting to note that the ID 50 of 7-ketocholesterol was approximately 10 times higher for cantharidin, an epidermal irritant, than for croton oil, a vascular irritant [12]. This indicates that 7-ketocholesterol had more potent activity against a vascular irritant than against an epidermal irritant, suggesting possible vasoconstrictor activity, a property of glucocorticoids.

Oxidized sterols have been noted to inhibit cellular DNA synthesis [13] and keratinocyte proliferation [13] in cell-culture systems, so it is surprising that 7-ketocholesterol had relatively less activity against the more potent hyperplasia-inducing irritant cantharidin [9].

There are few published studies that examine the anti-inflammatory potential of oxidized sterols. Using an in vitro assay, Gordon et al have demonstrated a possible explanation for the observed pharmacologic properties of oxidized sterols [6]. In this study, PML pre-incubated with oxidized sterols migrated poorly to a chemotactic stimulus, although random migration was normal. Since PML play a prominent role in the development of an irritant dermatitis [14], it is conceivable that topically applied 7-ketocholesterol may have interfered with the migration of PML to damaged skin, thus decreasing the degree of inflammation.

Histologic evaluation of inflamed skin (treated with 7-ketocholesterol) did demonstrate a suppression of the pathologic changes in the irritated skin. Most notably, there was a decrease in dermal edema and a thinning of the inflammatory infiltrate. Although this is not conclusive evidence that 7-ketocholesterol inhibited the migration of PML, it is not incompatible with this concept. Most likely, 7-ketocholesterol will influence multiple aspects of an inflammatory response, as do other anti-phlogistic agents [15].

Glucocorticoids have been noted to induce lympholysis, thymic atrophy, and shrinkage of lymph nodes and spleen in steroid-sensitive animals (i.e., mouse, rat, rabbit) but not in humans and other steroid-resistant species [15]. Only topically applied hydrocortisone had any significant systemic effects as measured by premature involution of the thymus gland. There was no suggestion of a dose-dependent induction of thymic involution by topical hydrocortisone. This implies that the topical glucocorticoid was applied in doses much higher than is necessary to induce dose-dependent thymic involution.

Oxidized sterols have been noted to inhibit sterol synthesis and proliferation of resting and mitogen-stimulated thymocytes [16], but there is no evidence to suggest that they will specifically lyse these cells in vitro or in vivo, as will glucocorticoids.

It is possible to infer that the lack of thymolytic activity by 7-ketocholesterol was related to poor systemic absorption. This possibility is unlikely, since we have demonstrated from previous experiments that intraperitoneal (i.e., systemic) doses of 7-ketocholesterol did not induce thymic involution (unpublished data). It is likely, then, that 7-ketocholesterol does not have the ability to lyse lymphatic tissue, as do glucocorticoids.

This study demonstrates that 7-ketocholesterol has weak anti-inflammatory properties; unlike glucocorticoids, it is not thymolytic. Since 7-ketocholesterol is but a single member of a large family of oxidized sterols [1], surely these compounds deserve further study to elucidate their structure–activity relationships. Furthermore, oxidized sterols are potent immunosuppressive [4] and antimitotic [5] molecules in cell-culture systems. These properties warrant further investigation using

animal models in order to characterize the pharmacologic and toxicologic properties of these cholesterol photoproducts.

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