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Effect of squalene on superoxide anion generation induced by a skin irritant, lauroylsarcosine

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Abstract

The effect of squalene on superoxide anion (O_2^-) generation was studied in rats in order to elucidate the mechanism whereby the compound decreases erythema induced by 1% lauroylsarcosine (LS) ointment. Topical application of superoxide dismutase (SOD) ointment (99000 U/g) was effective on the erythema as well as 10% squalene ointment, suggesting that O_2^- is a major mediator responsible for skin irritation. LS (200–400 μg/ml) caused overt production of O_2^- from cultured keratinocytes and peritoneal exudate leukocytes; O_2^- was significantly reduced by addition of squalene (100 μg/ml). A similar response was observed with zymosan-induced O_2^- generation. However, squalene had no effects on O_2^- generation in a xanthine-xanthine oxidase system unlike the effect observed with SOD. These results suggest that a possible role of squalene for the alleviation of skin irritation is suppression of O_2^- production depending on the different mechanism of action from SOD.

Keywords: Skin irritation; Superoxide anion; Squalene; Suppression; Rat

1. Introduction

Transdermal patches have been established as useful drug delivery systems maintaining an effective steady state in the blood level. However, there is a problem to overcome for further developments of this new type of drug delivery system: the lack of skin penetration of drugs. Enhances of transdermal absorption are considered to be available to improve skin penetration (Aungst et al., 1986; Chien, 1987).

Previously, we demonstrated that lauroylsarcosine (LS), a transdermal absorption enhancer, induced dose-related skin irritation following application onto rat dorsal skin and that squalene (Fig. 1) minimized this skin irritation (Aioi et al., 1993). Moreover, the combined addition of squa-

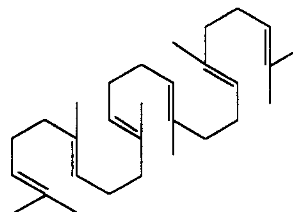

Fig. 1. Structure of squalene.

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lene and LS to isosorbide dinitrate ointment was demonstrated to maintain greater penetration and less irritation. In this paper, the effect of squalene on the generation of superoxide anion (O_2^-) has been studied to elucidate the mechanism whereby the compound alleviates the erythema in response to LS.

2. Materials and methods

2.1. Animals

Wistar male rats purchased from Japan SLC, Inc. (Hamamatsu, Japan) were subjected to investigation after housing for 7–10 days at constant temperature (24 ± 2°C) and relative humidity (55 ± 5%), under specific pathogen-free conditions with free access to food and water.

2.2. Preparation of ointment

Ointment containing LS (extra pure, Nacarai tesque, Kyoto, Japan) squalene (> 98% pure, Sigma Chemical, St. Louis, U.S.A.) or superoxide dismutase (SOD) derived from bovine erythrocytes (3300 U/mg, Wako Pure chemical, Osaka, Japan) was prepared using hydrocarbon gel (Taisho Pharmaceutical Co., Tokyo, Japan) as a base. Thus, each compound was added to hydrocarbon gel at an appropriate dose, with mixing in a mortar. The ointment was prepared just prior to use.

2.3. Skin irritation testing

0.1 g of ointment containing LS, squalene or SOD was mounted on a round polymer film (a laminated film of polyethyleneterephthalate and ethylenevinyl acetate, 3.14 cm^2), applied onto the shaved back of a rat weighing 150–180 g and fixed with gauze and bandage. After 24 h, the ointment was removed and the score of erythema was determined according to the method of Draize (1959) as follows: 1, mild erythema; 2, moderate erythema; 3, severe erythema.

2.4. Preparation of keratinocytes

Keratinocytes were prepared from shaved dorsal skin of rats weighing 30–50 g. The isolated skin was washed in phosphate-buffered saline at pH 7.2 (PBS) after cutting into fragments of approx. 2 cm^2. The skin fragments were incubated in 0.25% trypsin dissolved in PBS for 18 h at 4°C. Then, they were further incubated for 2 h at 37°C to peel off the epidermis. This epidermal sheet was stirred gently in PBS for 10 min at 37°C to separate keratinocytes. The separated keratinocytes were collected by centrifugation at 650 $\times g$ for 5 min and suspended in calcium-free Hank's balanced solution (HBSS) to contain 5×10^6 cells/ml (Indo, 1977; Furukawa, 1989).

2.5. Preparation of exudate leukocytes

Peritoneal exudate leukocytes were obtained from rats weighing 150–180 g. The rats were decapitated 16 h after an intraperitoneal injection (10 ml) of 0.2% casein in saline. Peritoneal leukocytes were harvested following an injection of 15 ml HBSS into the abdominal cavity. The cell suspension was centrifuged at $650 \times g$ for 5 min. The packed cells were rinsed three times and resuspended at a density of 5×10^6 cells/ml in HBSS. The majority of cells were polymorphonuclear leukocytes (Johnson, 1981).

2.6. Measurement of O_2^- generation

The amount of O_2^- was determined by measuring the reduction of equine ferricytochrome *c* (McCord and Fridovich, 1969). In this assay, each compound was dissolved or suspended in HBSS. The cell suspension (400 μl) of keratinocytes or leukocytes was mixed with 20 mM $CaCl_2$ (50 μl), 10 mM ferricytochrome *c* (100 μl), squalene or SOD (50 μl) and LS or opsonized zymosan (Markert et al., 1984; Bird and Giroud, 1985) (400μl). The mixture was incubated at 37°C for 10 min (leukocytes) or 30 min (keratinocytes) and the absorbance was monitored at 550 nm. The amount of O_2^- generated was calculated based on the difference in absorbance from the reference

using an absorbance coefficient of 22.6 mM^{-1} cm^{-1}.

O_2^- generation in the xanthine-xanthine oxidase system was assayed as follows. 10 mM ferricytochrome *c* (100 μl) was mixed with 5 mM xanthine (100 μl), 160 U/ml xanthine oxidase (200 μl) and squalene or SOD (600 μl). After incubation for 10 min at 37°C, the amount of O_2^- generated was measured as mentioned above.

2.7. Statistical analysis

The values given as the mean ± S.D. were statistically analyzed using Student's *t*-test.

3. Results

3.1. Effects of squalene and SOD on LS-induced erythema

Squalene and SOD were applied onto dorsal rat skin together with LS to evaluate their effects on skin irritation. As reported previously (Aioi et al., 1993), LS produces a dose-dependent irritation on rat skin at doses of 0.04–5%. The compound induces erythema at low doses, concomitantly with edema at higher doses. In this study, 1% LS ointment was applied to induce erythema only. All animals dosed with 1% LS ointment showed erythema of score 2. Ointment containing SOD (99000 U/g) was effective as well as 10% squalene ointment. Thus, SOD or squalene decreased the erythema score from 2 to 1 in four or five of six animals, respectively Table 1. This suggests an involvement of O_2^- in LS-induced erythema.

Table 1
Effects of squalene and SOD on lauroylsarcosine (LS)-induced erythema

Compound	Responders/total	
	Score 1	Score 2
1% LS	0/6	6/6
1% LS + 10% squalene	5/6	1/6
1% LS + 90000 U/g SOD	4/6	2/6

Squalene or SOD was applied together with LS. Each value indicates the result of six animals.

3.2. Effect of squalene on LS-induced O_2^- generation from keratinocytes and leukocytes

The effect on O_2^- generation was estimated using cultured keratinocytes and peritoneal exudate leukocytes. The result indicated that 200 or 400 μg/ml of LS was the appropriate concentration for keratinocyte or leukocyte assay, respectively. The effect of squalene is shown in Table 2. The compound resulted in a marked inhibitory effect at 100 μg/ml for keratinocytes and at 25 μg/ml for leukocytes.

The addition of LS to cultured keratinocytes produced O_2^- at a rate of 0.66 μmol/min. This

Table 2
Effect of squalene on O_2 generation from cultured keratinocytes and peritoneal exudate leukocytes in rats

Inducer	Cell	Compound	Amount of O_2^- generated (μmol/min)
Lauroylsarcosine	keratinocytes	control	0.66 ± 0.08
(LS)		squalene 100 μg/ml	0.35 ± 0.06 [b]
(200 μg/ml)		SOD 330 U/ml	0.22 ± 0.10 [b]
LS	leukocytes	control	1.15 ± 0.56
(400 μg/ml)		squalene 25 μg/ml	0.69 ± 0.40 [a]
		SOD 330 U/ml	0.24 ± 0.04 [a]
Opsonized	leukocytes	control	0.62 ± 0.12
zymosan		squalene 25 μg/ml	0.35 ± 0.10 [b]
(2 mg/ml)			

Each value indicates the mean ± S.D. of five experiments. [a] $p < 0.01$, [b] $p < 0.001$: significant relative to the control using Student's *t*-test.

O_2^- production was significantly inhibited by addition of squalene (100 mg/ml), although the activity was less potent than that of SOD (330 U/ml). A similar effect was also observed for peritoneal exudate leukocytes. Squalene (25 μg/ml) reduced LS-induced O_2^- generation from 1.15 to 0.69 μmol/min. In addition, in an experiment on opsonized zymosan, frequently used as a model for the evaluation of anti-inflammatory agents (Markert et al., 1984), the level of O_2^- rose 0.62 μmol/min on the addition of opsonized zymosan (2 mg/ml) and squalene reduced this level to 0.35 μmol/min.

3.3. *Effect of squalene on O_2^- generation in a cell-free system*

The effect of squalene on O_2^- generation was evaluated in a xanthine-xanthine oxidase system. In this model, SOD markedly depressed the rise in O_2^- level at low doses of 3.3 and 33 U/ml, consistent with other reports (Draize, 1959; Asada et al., 1974). However, squalene had no effects at 25 μg/ml and even 100 μg/ml at which O_2^- generation from keratinocytes and leukocytes was reduced (Table 3).

4. Discussion

The results presented here suggest that O_2^- is a major mediator responsible for LS-induced erythema. In addition, squalene, an alleviator of skin irritation, suppressed the production of O_2^- from cultured keratinocytes and peritoneal exudate leukocytes following LS exposure.

Reactive oxygen species have been studied extensively and are believed to be closely related with pathogenic responses such as generation of lipid peroxidation (Tyler, 1975), canceration (Slaga and Bracken, 1977), atherosclerosis (Wilson et al., 1978) and inflammation (McCord, 1974). One reactive oxygen species, O_2^-, is well known to be an important bactericidal factor to maintain physiological homeostasis (Babior et al., 1975), however, the factor excessively produced in tissue causes peroxidation of unsaturated fatty acid (Thomas et al., 1978), release of lysosomal enzymes (Fong et al., 1973), biosynthesis of prostaglandins (Panganamala et al., 1974), alternation of vascular tone and microvascular permeability (Okabe et al., 1983; Silin et al., 1985) to initiate or/and promote inflammation. This is not an exception to the cutaneous response. Several studies have shown that reactive oxygen species including O_2^- are probably involved in several inflammatory skin diseases such as systemic lupus erythematosus (Sakane et al., 1985), psoriaris (Sedgwick et al., 1981), contact dermatitis (Miyachi et al., 1987) and sunburn injury (Miyachi et al., 1983). In this paper, we also demonstrated that SOD apparently alleviated LS-induced erythema, indicating a possible role of O_2^- in a complex process of erythema appearance.

Previously, we reported that skin irritation of LS is microscopically characterized by keratinocyte damage and infiltration with polymorphonuclear leukocytes (Aioi et al., 1993). Based on these findings, this experiment was performed to elucidate the effect of squalene on the modulation of keratinocytes and leukocytes. The results of in vitro assay using cultured keratinocytes and peritoneal exudate leukocytes showed that O_2^- was produced by additions of LS to the both cells and opsonized zymosan to leukocytes and that squalene depressed such O_2^- production. However, there was a clear difference in the mode of action between squalene and SOD. The evidence was provided by the experiment on the xanthine-xanthine oxidase system, which demonstrated that O_2^- generated following the xanthine oxidase re-

Table 3
Effect of squalene on O_2^- generation in a xanthine-xanthine oxidase system

Compound	Dose	Amount of O_2^- generated (μmol/min)
Squalene	control	4.49 ± 0.42
	25 μg/ml	4.16 ± 0.30
	100 μg/ml	4.37 ± 0.34
SOD	control	4.97 ± 0.34
	3.3 U/ml	2.03 ± 0.34 [a]
	33 U/ml	0.91 ± 0.22 [a]

Each value indicates the mean ± S.D. of five experiments.
[a] $p < 0.001$: significant relative to the control using Student's *t*-test.

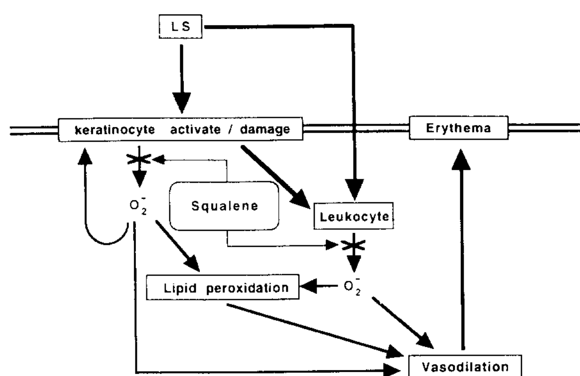

Fig. 2. Hypothetical scheme for O_2^- generation during LS-induced erythema and action of squalene.

action was scavenged by SOD but not by squalene. These findings indicate that squalene may depress O_2^- generation during LS-induced inflammation via a different mechanism of action from SOD and alleviate the erythema depending on this activity. Although the detailed mechanism of action of squalene is unclear, it might be similar to that of vitamin E which attenuates O_2^- production by stimulated macrophages through interaction with the cell membrane (Sakamoto et al., 1991). This is because both compounds share some physico-chemical properties in common such as lipophilicity and are telepenoids.

From the above, a possible role of squalene in the alleviation of LS-induced skin irritation is postulated to be the suppression of O_2^- production as shown in Fig. 2. However, since SOD could incompletely inhibit LS-induced erythema, it could not be excluded that squalene might also act on other inflammatory pathways related with interleukin-1 and prostaglandins (Kupper, 1989).

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