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Effects of 12-*O*-Tetradecanoylphorbol-13-acetate and Mezerein on Epidermal Ornithine Decarboxylase Activity, Isoproterenol-stimulated Levels of Cyclic Adenosine 3′:5′-Monophosphate, and Induction of Mouse Skin Tumors *in Vivo*[1]

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ABSTRACT

The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate and the antileukemic agent mezerein are diterpene esters of plant origin with certain structural similarities. Both compounds, when applied topically to mouse skin, were equipotent on a molar basis in inducing hyperplasia, inflammation, and ornithine decarboxylase activity, as well as in reducing cyclic adenosine 3′:5′-monophosphate accumulation in response to β-adrenergic stimulation. In contrast, mezerein was much less effective as a tumor promoter; the phorbol ester at 8.5 nmol/application yielded 78-fold more tumors than did 8.5 nmol mezerein per application to similarly initiated SENCAR mice. The superiority of the phorbol ester was nearly as great in CD-1 mice.

These results suggest that although the induction of hyperplasia and ornithine decarboxylase activity may be necessary components of the carcinogenic process, they are not sufficient; 12-*O*-tetradecanoylphorbol-13-acetate must accomplish an essential event not accomplished by mezerein.

INTRODUCTION

Mezerein, a plant-derived ester of 12-hydroxydaphnetoxin, has significant antileukemic activity against P-388 and L1210 mouse leukemias (16, 21). Chemical and crystallographic analyses of mezerein have revealed a striking structural similarity to the mouse skin tumor promoter TPA,[4] the most potent tumor promoter of the series of phorbol esters isolated from croton oil (3, 16, 21). Both compounds are diterpenes, contain cyclopentenone rings, and possess long-chain lipophilic groups esterified at position 12 (Chart 1). The free alcohol phorbol is inactive as a tumor promoter, and the unesterified 12-hydroxydaphnetoxin is inactive as an antileukemic agent (21).

Recent studies of the effects of mezerein on cells in culture have revealed that mezerein and TPA elicit many of the same responses. Mezerein is nearly equipotent on a molar basis with TPA in acting as a comitogen in phytohemagglutinin-treated bovine lymphocytes (15). Mezerein is approximately 5 times as effective as TPA in inducing plasminogen activator in chick embryo fibroblasts and is almost as effective as TPA in inhibiting differentiation of murine erythroleukemia cells (32), chick embryonic ganglion cells (13), and murine neuroblastoma cells (14). Furthermore, mezerein is also as effective as TPA in inhibiting the binding of epidermal growth factor to HeLa cells (17).

It has also been reported that mezerein is an irritant for mouse skin, as are the phorbol esters (7). The application of TPA to mouse skin results in diverse morphological and biochemical responses. Among the most dramatic are tumor promotion (2, 3, 9), induction of inflammation and hyperplasia (3, 9, 24, 25, 30), induction of ODC activity (22, 23), and reduction in cAMP accumulation in response to β-adrenergic stimulation (8, 19, 20). Since mezerein is structurally similar to TPA, has irritant properties, and produces TPA-like effects on cells in culture, we have investigated effects of mezerein on various parameters associated with the tumor-promoting activity of TPA.

MATERIALS AND METHODS

Materials. Female Charles River CD-1 mice or female Oak Ridge SENCAR mice were used at 7 to 9 weeks of age. DL-Isoproterenol hydrochloride, β-retinoic acid, the sodium salt of cAMP, and calf thymus DNA (type 1) were purchased from Sigma Chemical Co., St. Louis, Mo. Dowex AG 50W-X4 H^+ (200 to 400 mesh) cation-exchange resin was from Bio-Rad Laboratories, Richmond, Calif. TPA was purchased from both Consolidated Midland Corp., Brewster, N. Y., and Dr. Peter Borchert, University of Minnesota, Minneapolis, Minn. Mezerein was a gift from the late Dr. S. M. Kupchan, University of Virginia, Charlottesville, Va. DL-[1-^{14}C]Ornithine hydrochloride (specific activity, 49.9 mCi/mmol) and [^{3}H]cAMP (specific activity, 37 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. The solutions of TPA and mezerein were prepared in acetone. Acetone solution of TPA was stored in the dark at −20°, but mezerein solution was freshly prepared before use.

Treatment of Mice. Mice were housed 10/cage, and food and water were available *ad libitum*. The dorsal skin was shaved 3 to 4 days before experimentation, and only those mice not showing hair regrowth over this period were used. Acetone solutions of TPA or mezerein were applied to the shaved areas of individual mice in a volume of 0.2 ml. Control mice were treated with the same volume of acetone.

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[4] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ODC, ornithine decarboxylase (EC 4.1.1.17); cAMP, cyclic adenosine 3′:5′-monophosphate; DMBA, 7,12-dimethylbenz(*a*)anthracene.

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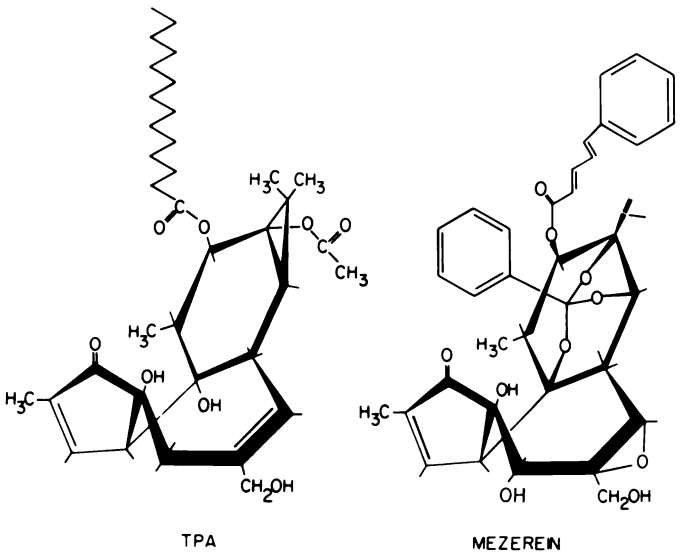

Chart 1. Structures of TPA and mezerein.

Tumor Induction Experiments. Mice were initiated by application of 0.1 μmol DMBA in 0.2 ml acetone. Following initiation, all mice were promoted twice a week with either TPA or mezerein for the duration of the experiment. There were at least 30 mice in each treatment group. The incidence of papillomas was observed weekly.

Histological Examinations. The number of nucleated interfollicular epidermal cell layers and the number of inflammatory cells in the dermis of CD-1 mice were measured in 5-μm-thick sections of skin stained with hematoxylin and eosin using a modification of the procedure described by Raick (24). TPA at doses of 3.4 and 17 nmol and mezerein at doses of 0.34 and 3.4 nmol were used to determine the inflammatory and hyperplastic response to these compounds.

cAMP Determinations. The dorsal skin from cervically dislocated animals was immediately frozen on dry ice (−70°). Pure epidermal preparations were scraped off and immediately homogenized in cold 5% trichloroacetic acid with a Polytron PT10. cAMP was purified from the ether-extracted acid-soluble material on columns of Dowex AG 50W-X4 H^+ (200 to 400 mesh) as previously described (18). cAMP content was determined by the protein-binding assay previously reported (5). DNA content was determined by the diphenylamine method of Burton (6).

Assay of ODC Activity. At appropriate times after treatment, mice were killed by cervical dislocation, and the epidermis from individual mice was separated by a brief heat treatment (55° for 30 sec). The epidermal preparations from 2 to 3 mice were pooled, homogenized in 50 mM sodium phosphate buffer (pH 7.2) containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA, and centrifuged at 30,000 × *g* for 30 min to give a soluble supernatant.

ODC activity in the clear supernatant was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine hydrochloride. The assay mixture contained 35 mM sodium phosphate (pH 7.2), 0.2 mM pyridoxal phosphate, 4 mM dithiothreitol, 1 mM EDTA, 0.4 mM L-ornithine containing 0.5 μCi of DL-[1-^{14}C]ornithine hydrochloride, and 100 μl of epidermal extract in a final volume of 0.25 ml. Assays were always carried out in duplicate; blank assays contained no enzyme or boiled enzyme. The incubation and radioactive counting conditions were as previously described (28). Protein content from the epidermal extracts was determined by the procedure of Lowry *et al.* (18).

RESULTS

Effects of TPA and Mezerein on Inflammation and Epidermal Cell Proliferation. The effect of single applications of TPA or mezerein on the number of nucleated interfollicular epidermal cell layers is presented in Table 1. Both treatments produced epidermal hyperplasia within 1 day following treatment; a 2- to 3-fold increase in the number of nucleated cell layers was observed 2 to 3 days after application. A comparison of the effect of the 3.4-nmol doses of TPA and mezerein reveals that mezerein produced a more sustained hyperplasia than did TPA. Both agents caused inflammation, as determined histologically by the presence of edema, and infiltration of reticuloendothelial cells into the dermis.

Effects of TPA and Mezerein on ODC Induction. A time course of the effect of topical application of either TPA or mezerein (3.4 nmol each) on mouse epidermal ODC activity is shown in Chart 2. Treatment with either TPA or mezerein led to a pronounced increase (about 300-fold) in the activity of ODC; the peak activity occurred between 4 and 8 hr, and the enzyme activity declined to the basal level at about 12 hr following

Table 1

Effects of a single topical application of various doses of TPA and mezerein on the number of nucleated interfollicular layers of CD-1 mouse skin

Mice were treated with various doses of TPA or mezerein. The number of nucleated interfollicular cell layers and the inflammatory index were determined on 5-μm-thick sections of the skin as described in "Materials and Methods." Each value represents 7 observations (S.D. <20%).

Treatment	Dose (nmol)	No. of nucleated interfollicular layers after treatment				Inflammatory index after treatment	
		Day 1	Day 2	Day 3	Day 4	Day 1	Day 2
Acetone		1.3	1.5	1.3	1.3	0	0
TPA	17	3.4	3.6	4.8	5.6	4	4
TPA	3.4	2.3	3.4	3.6	2.1	2	2
Mezerein	3.4	3.6	4.0	4.0	3.5	3	3
Mezerein	0.34	2.0	1.9	1.5	1.3	0	0

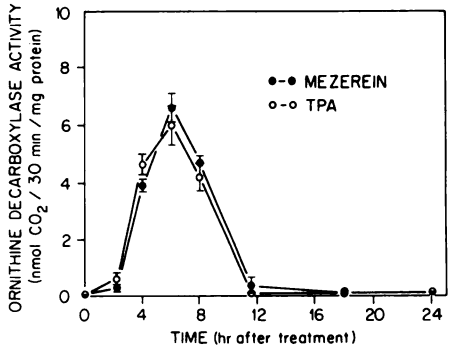

Chart 2. Effect of treatment with TPA or mezerein on mouse epidermal ODC activity. Groups of mice were treated with 0.2-ml acetone solutions of either 3.4 nmol TPA or 3.4 nmol mezerein. Mice were killed for enzyme assay at the indicated time. Each *point* represents the mean of determinations of ODC activity from 3 groups of mice, and each group contained the combined soluble epidermal extracts prepared from 3 mice; *bars*, S.E.

treatment with either compound. Both compounds exhibited identical time courses and a similar degree of induction of epidermal ODC activity following treatment with a 3.4-nmol dose.

A comparison between the effect of various doses of TPA or mezerein on induction of epidermal ODC activity is shown in Table 2. No significant ($p < 0.1$) difference in the degree of induction of ODC activity was observed at any of the doses tested.

Vitamin A acid (retinoic acid) has been shown to be a potent inhibitor of TPA-induced mouse epidermal ODC activity (27). It was of interest to determine whether retinoic acid treatment could inhibit the increase in ODC activity by mezerein. Results shown in Table 3 indicate that 1.7 nmol retinoic acid applied 1 hr prior to treatment with 3.4 nmol mezerein decreased (86%) ODC induction as effectively as when applied before TPA treatment.

Effects of TPA and Mezerein on cAMP Accumulation. It has been previously demonstrated (4) that 13 hr after TPA application to mouse skin the accumulation of cAMP in response to β-adrenergic stimulation is dramatically reduced. The basal cAMP level at this time is, however, unaltered. As indicated in Table 3, mezerein alone also has no effect on the basal level of cAMP in mouse epidermis; however, mezerein and TPA were approximately equipotent at 17-nmol doses in reducing the accumulation of cAMP in response to i.p. injection of 300 nmol (−)-isoproterenol (Table 4).

Skin Tumor Promotion by TPA or Mezerein. The effect of TPA as a potent tumor promoter has been reported previously (1, 2, 11). The promoting activity of various doses of mezerein and TPA in CD-1 mice initiated with 200 nmol DMBA is given in Table 5. Promotion with 1.7 nmol TPA resulted in 1.2 papillomas/mouse, whereas the same promotion regimen with mezerein produced no tumors. A 17-nmol dose of mezerein, however, resulted in 0.3 papillomas/mouse. In a separate experiment, 0.34 and 3.4 nmol mezerein were devoid of promoting activity in CD-1 mice. In contrast, application of 0.34 and 3.4 nmol TPA resulted in 0.3 and 5.6 papillomas/mouse, respectively.

SENCAR mice are more susceptible to tumorigenesis by chemical carcinogens than are CD-1 mice. The results of a tumor experiment in SENCAR mice initiated with 100 nmol DMBA and promoted with either TPA or mezerein are shown in Table 6. Application of 3.4 and 17 nmol mezerein produced 1 and 1.7 papillomas/mouse, respectively, whereas 1.7 nmol TPA resulted in 8.6 papillomas/mouse. In a separate experiment, application of 8.5 nmol TPA resulted in 78-fold more tumors than did 8.5 nmol mezerein. A more detailed study of various doses of TPA and mezerein on formation of skin papillomas in SENCAR mice was made. Application of 3.4 nmol TPA yielded 20.1 papillomas/mouse as compared to 9.3 for 1.7 nmol TPA and 1.9 for 17 nmol mezerein.

Table 2

Effect of the dose of TPA or mezerein on mouse epidermal ODC activity

Mice were treated with 0.2-ml acetone solutions of either various doses of TPA or mezerein, and soluble epidermal extracts were prepared 4.5 hr after treatment. Each value represents the mean ± S.E. of determinations of enzyme assay from 3 groups of mice; each group contained the combined supernatant prepared from 3 mice.

	ODC activity after treatment with the following doses (nmol CO_2/30 min/mg protein)			
Treatment	0.34 nmol	1.70 nmol	3.40 nmol	17.0 nmol
TPA	1.08 ± 0.33	4.8 ± 0.45	5.41 ± 0.44	6.36 ± 1.24
Mezerein	0.71 ± 0.18	3.01 ± 0.48	5.08 ± 0.29	8.25 ± 0.21

Table 3

Effect of pretreatment with retinoic acid on TPA- or mezerein-induced mouse epidermal ODC activity

Groups of mice were treated with 1.7 nmol retinoic acid in 0.2 ml acetone 1 hr prior to treatment with either 0.2 ml acetone solution of 3.4 nmol mezerein or 3.4 nmol TPA. Mice were killed for enzyme assay 4.5 hr after treatment with mezerein or TPA. Each value represents the mean ± S.E. of determination of enzyme assay from 3 groups of mice with 3 mice/group.

Treatment	ODC activity (nmol CO_2/30 min/mg protein)
Acetone-acetone	0.02
Acetone-TPA	5.41 ± 0.44
Retinoic acid-TPA	0.67 ± 0.12
Acetone-mezerein	5.19 ± 1.09
Retinoic acid-mezerein	0.74 ± 0.12

Table 4

The effect of mezerein and TPA on the isoproterenol-stimulated levels of cAMP in mouse epidermis

Thirteen hr prior to sacrifice, mice were treated topically as indicated below; 10 min prior to sacrifice, they were given injections of either 0.9% NaCl solution or isoproterenol. After sacrifice, snap-frozen epidermis was collected and analyzed for cAMP and DNA.

Treatment		Experiment 1 (pmol cAMP/μg DNA)	Experiment 2 (pmol cAMP/μg DNA)
Topical	i.p.		
Acetone	0.9% NaCl solution	0.49 ± 0.15[a]	0.32 ± 0.03
Mezerein (17 nmol)	0.9% NaCl solution	0.42 ± 0.08	0.32 ± 0.05
Acetone	(−)-Isoproterenol (300 nmol)	7.35 ± 1.98	9.40 ± 1.30
TPA (17 nmol)	(−)-Isoproterenol (300 nmol)	1.18 ± 0.58	1.95 ± 0.41
Mezerein (17 nmol)	(−)-Isoproterenol (300 nmol)	1.49 ± 0.55	0.87 ± 0.21

[a] Mean ± S.E.

Table 5

Comparison of the effectiveness of TPA and mezerein as promoters of skin tumorigenesis in CD-1 mice

The mice in Experiment 1 were initiated with 200 nmol DMBA and promoted 10 days later with twice-weekly applications of either TPA or mezerein at the indicated dose. The mice in Experiment 2 were also initiated as above but were promoted 7 days later with twice-weekly applications of TPA or mezerein.

Promoter Dose (nmol)	Total no. of surviving mice at 20 wk	Papillomas/mouse at 20 wk	% of mice with papillomas at 20 wk
Experiment 1			
Acetone	27	0	0
TPA 1.7	29	1.2	64
TPA 17	27	7.1	93
Mezerein 1.7	27	0	0
Mezerein 17	23	0.3	23
Experiment 2			
Acetone	29	0	0
TPA 0.34	29	0.3	21
TPA 3.4	29	5.6	75
Mezerein 0.34	29	0	0
Mezerein 3.4	27	0	0

DISCUSSION

TPA and mezerein are both plant-derived diterpene esters with some structural similarity (Chart 1). The structure-activity relationships for these and many other related irritants have

Table 6

Comparison of the effectiveness of TPA and mezerein as promoters of skin tumorigenesis in SENCAR mice

The mice in Experiments 1 and 2 were initiated with 100 and 200 nmol DMBA, respectively, and promoted 1 week later with twice- or 3 times-weekly applications of mezerein or TPA.

Promoter Dose (nmol)	Total no. of surviving mice at 16 wk	Papillomas/ mouse at 20 wk	% of mice with papillomas at 20 wk
Experiment 1			
Acetone	29	0	0
TPA 1.7	28	8.6	100
Mezerein 3.4	27	1.0	38
Mezerein 8.5	26	1.2	48
Mezerein 17	25	1.7	62
Experiment 2			
Acetone	28	0	0
TPA 8.5	27	15.6	100
Mezerein 8.5[a]	25	0.2	10

[a] Mezerein was given 3 times/week at a dose of 8.5 nmol/application.

been investigated and reviewed in detail by Hecker (9). The relationship between the inflammatory and hyperplastic properties of various irritants to their tumor-promoting activity has been a matter of speculation (12). Slaga *et al.* (26) have shown a correlation between tumor-promoting activity of phorbol esters and their hyperplasiogenic activity in mouse epidermis. However, Scribner and Boutwell (25) have suggested that there is no relationship between the inflammatory and tumor-promoting activity of the phorbol esters. The data presented here demonstrate that, although mezerein is at least as potent as TPA on an equimolar basis in increasing the number of nucleated interfollicular epidermal cell layers and in increasing the number of reticuloendothelial cells in the dermis, it is a very weak tumor promoter.

Hecker (9) has reported the weak tumor-promoting activity of mezerein in NMRI mice and showed that it was less than 4% as active as TPA. The data presented here confirm the weak promoting ability of mezerein. Application of 1.7 nmol TPA produced 5 times more tumors than did 17 nmol mezerein in SENCAR mice, and 17 nmol TPA produced almost 25 times as many papillomas/mouse as did 17 nmol mezerein in CD-1 mice. Since mezerein is as hyperplasiogenic as TPA, the correlation between hyperplasiogenic activity and tumor-promoting activity developed for the phorbol esters (26) does not extend to esters of 12-hydroxydaphnetoxin, a related diterpene. Furthermore, since mezerein also possesses inflammatory activity equivalent to TPA but is a much weaker tumor promoter, inflammatory activity can clearly be dissociated from tumor-promoting activity. This has been previously demonstrated with a series of phorbol esters by Scribner and Boutwell (25).

Both the induction of ODC and reduction of β-adrenergic accumulation of cAMP have been shown to correlate with tumor-promoting activity of phorbol esters (8, 9, 20, 23). These phenomena have also been associated with transformation and rapid cell growth in other systems (31). Furthermore, the induction of ODC has been suggested to be closely related to tumor promotion in mouse skin (29). It is, therefore, interesting that mezerein, a relatively weak mouse skin tumor promoter, can induce ODC and reduce β-adrenergic responsiveness to the same extent as can TPA.

The results presented indicate that although mezerein is as potent as TPA in its ability to induce hyperplasia and ODC activity and to depress isoproterenol-stimulated cAMP level, it is a very weak tumor promoter. This suggests that induction of ODC activity as well as the decrease in cAMP accumulation in response to the agonist isoproterenol may be necessary components of the mechanism of skin tumor promotion, but they are not sufficient components. By deduction, TPA must accomplish an essential event not accomplished by mezerein. A further study of the effects of several other compounds structurally related to TPA (10) may help to understand the complex mechanism of action of skin tumor promotion by TPA.

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