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α-Difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, inhibits tumor promoter-induced polyamine accumulation and carcinogenesis in mouse skin

(putrescine/decarboxylation inhibitor/stage-specific promotion/tumorigenesis/phorbol diester)

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ABSTRACT **The role of ornithine decarboxylase (OrnDCase, EC 4.1.1.17) and of the polyamines [putrescine (Put), spermidine (Spd), and spermine (Spm)] in mouse skin tumor promotion was investigated by the use of α-difluoromethylornithine (CHF_2-Orn), an enzyme-activated irreversible inhibitor of OrnDCase. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), mezerein, and ethyl phenylpropiolate (EPP) were employed as complete, stage II specific, and nonpromoting agents, respectively. TPA and mezerein, but not EPP, provided for a dose-dependent increase in tissue Put accumulation. The Put level in papillomas developed by TPA (2 μg) treatment was ≈15-fold higher than that of the surrounding skin tissue; Spd accumulation was 2- to 3-fold greater in the papillomas. Put administered (intraperitoneally) with TPA greatly enhanced papilloma yield. CHF_2-Orn, given orally or intraperitoneally, abolished the TPA-induced OrnDCase activity and Put accumulation in mouse epidermis. The reduction of polyamine accumulation by CHF_2-Orn was directly proportional to reduction of tumor size. CHF_2-Orn administered in a two-stage (TPA–mezerein) promotion protocol [Slaga, T. J., Fischer, S. M., Nelson, K. G. & Gleason, G. L. (1980) *Proc. Natl. Acad. Sci. USA* 77, 3659–3663; Slaga, T. J., Klein-Szanto, A. J. P., Fischer, S. M., Weeks, C. E., Nelson, K. & Major, S. (1980) *Proc. Natl. Acad. Sci. USA* 77, 2251–2254] reduced tumor size, inhibited by 65–70% the number of papillomas per mouse, and decreased by 40% the percentage of mice with tumors when given with the stage II agent mezerein. CHF_2-Orn provided considerably less effect on tumorigenesis when administered with the TPA portion of the protocol, and CHF_2-Orn did not inhibit the induction of dark basal keratinocytes by TPA. Based on our results with CHF_2-Orn, we suggest that regulation of polyamine biosynthesis, particularly Put, is a critical factor in stage II promotion.**

Carcinogenesis in mouse skin can be induced by the sequential application of a subthreshold dose of a carcinogen (initiation phase) followed by repetitive treatment with a noncarcinogenic tumor promoter. The initiation phase requires only a single application of a carcinogen and is essentially irreversible. The promotion phase is initially reversible but later becomes irreversible (1). Recent data from our laboratory suggest that the tumor promotion stage can be divided into two stages (2–5). The first stage can be effectively brought about by limited applications of 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Mezerein, a diterpene similar to TPA in its biochemical and morphological effects, is a weak promoter but is a potent stage II agent when given repetitively after stage I promotion (2–6). The nonpromoting, hyperplastic agent ethyl phenylpropiolate (EPP) provides neither stage response. EPP, like mezerein, does not induce dark basal keratinocytes (4, 7–9). However, TPA does and we have suggested that these embryonic-like basal cells are important in stage I promotion (3–5).

In addition to inducing "dark cells" and causing epidermal inflammation and hyperplasia, the phorbol ester tumor promoters have been shown to have many other effects on the skin and cells in culture (reviewed in refs. 1 and 10). It is therefore difficult to determine which of the many responses are essential components of the promotion process. O'Brien *et al.* (11) reported an excellent correlation between the tumor-promoting ability of various compounds (phorbol esters as well as nonphorbol ester compounds) and their ability to induce ornithine decarboxylase (OrnDCase; EC 4.1.1.17) activity in mouse skin. Recently, the response of enhanced OrnDCase activity has been established in other two-stage systems (12–14). Furthermore, vitamin A analogs (15) and inhibitors of prostaglandin biosynthesis (16) inhibit tumor promotion with the degree of effectiveness related to the potency of OrnDCase inhibition.

Additional information regarding the possible control of cellular development by the polyamines has been presented by our laboratory (17) and by others (18–21). Also blockage of polyamine biosynthesis by α-difluoromethylornithine (CHF_2-Orn) slows or arrests (*i*) intestinal mucosal maturation and injury recovery in rats (22), (*ii*) trypanosome growth and lethality (23), and (*iii*) mouse L-1210 leukemia progression (24). Many other reports have described the importance of polyamine levels in the control of cellular growth (reviewed in refs. 25–27). Our previous studies with CHF_2-Orn and other inhibitors of polyamine biosynthesis in the mouse skin two-stage tumorigenesis system demonstrated a correlation between polyamine level and tumor formation (10, 28, 29, †). We therefore used CHF_2-Orn in this study to further define the role of the polyamines in mouse skin tumor promotion. The effects of various classes or stage types of promoting agents were compared. Our results demonstrate that polyamine biosynthesis control relates directly to stage II of promotion.

MATERIALS AND METHODS

Chemicals. TPA and mezerein were purchased from Chemical Carcinogenesis (Minneapolis, MN). 7,12-Dimethylbenz[*a*]anthracene [$(CH_3)_2$BA] was obtained from Sigma. CHF_2-

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; $(CH_3)_2$BA, 7,12-dimethylbenz[*a*]anthracene; OrnDCase, ornithine decarboxylase; EPP, ethyl phenylpropiolate; CHF_2-Orn, α-difluoromethylornithine; Put, putrescine; Spd, spermidine; Spm, spermine; i.p., intraperitoneal(ly); P_i/NaCl, phosphate-buffered saline.

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† Weeks, C. E., Herrmann, A., Nelson, F. & Slaga, T. J. (1980) in *Cocarcinogenesis and Biological Effects of Tumor Promoters*, Symposia, Klais/Bavaria, Federal Republic of Germany, abstr. 101.

Orn was a generous gift from the Centre de Recherche Merrell International (Strasbourg, France) and from Merrell National Laboratories (Cincinnati, OH). DL-[1-^{14}C]ornithine (50 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) and S-adenosyl-L-[*carboxyl*-^{14}C]methionine (55 mCi/mmol) were purchased from New England Nuclear. EPP was obtained from Aldrich (Milwaukee, WI). Solvents of HPLC grade were from Burdick and Jackson (Muskegon, MI).

Assay of Ornithine and S-Adenosylmethionine Decarboxylase Activities. Procedures followed are those we reported earlier (17, 30).

Polyamine Analysis. Polyamine levels were quantitated via HPLC separation by using an internal standard calibration method after conversion to the dansyl derivatives as we previously reported (30).

Tumor Promotion Protocol. Female SENCAR mice, bred and housed in the Biology Division, Oak Ridge National Laboratory, were used for the experiments. The protocol followed for TPA promotion has been detailed elsewhere (31) as has that for two-stage (TPA–mezerein) promotion (3, 5). In the tumorigenesis experiments, mice were initiated with 10 or 100 nmol of $(CH_3)_2BA$ and beginning the following week they were given continuous biweekly topical applications of TPA (1 or 2 μg) or mezerein (2 μg) as required in 0.2 ml of acetone. CHF_2-Orn was administered to the mice via two routes. Groups receiving intraperitoneal (i.p.) injections (20 or 5 mg) were treated 2 hr prior to the biweekly TPA or mezerein application; control mice received similar 0.1-ml i.p. injections of phosphate-buffered saline (P_i/NaCl) solutions. Mice receiving topical TPA treatment were given CHF_2-Orn continuously in the drinking water; the concentration of CHF_2-Orn was adjusted as needed to provide for CHF_2-Orn levels between 1.5% and 2.0% (wt/vol) in the drinking water. At an average daily intake (mean ± SD) of 8.53 ± 0.89 ml per mouse per day, this provided for an intake (mean ± SD) of 4.7 ± 0.5 g/kg—a dose which does not exceed the reported LD_{50} (32)—for the daily average consumption over the 18-wk experimental period.

RESULTS AND DISCUSSION

The effects of one treatment of the complete promoter TPA, the stage II agent mezerein, and the very weak tumor promoter EPP on OrnDCase and polyamine levels in mouse epidermis are shown in Table 1. Doses applied caused similar hyperplastic responses (3, 7–9). TPA and mezerein enhanced OrnDCase activity 100- to 200-fold over control and provided for a dose-dependent increase in epidermal putrescine (Put) content. At 9 hr after treatment, 1.0, 2.0, and 5.0 μg of TPA provided 1.8, 2.9, and 3.7 nmol of Put per mg of protein, respectively. Similar doses of mezerein yielded 2.1, 2.7, and 5.5 nmol of Put per mg of protein. At 12 hr after mezerein application, Put levels were 3.3, 4.3, and 8.4 nmol/mg of protein at doses of 1.0, 2.0, and 5.0 μg, respectively. Both mezerein and TPA enhanced spermidine (Spd) levels in epidermis ≈2-fold between 12 and 48 hr after treatment. Although EPP at a dose of 3 mg enhanced OrnDCase activity about 25-fold over control, no significant increase in polyamine levels was seen. Likewise, a 3-fold or 10-fold higher dose of EPP did not increase the OrnDCase activity or Put level above that seen with the 3-mg dose level (data not shown). Similarly, mezerein and TPA appeared to depress spermine (Spm) quantity compared with control levels during the first hours after application, whereas EPP had no effect. Our results on OrnDCase activity fluctuations induced by TPA, mezerein, and EPP compare favorably to those reported (6, 11, 33, 34).

The Put level in papillomas and the effect of Put given i.p. on TPA promotion are shown in Table 2. Put significantly enhanced (>160%) tumor yield when given with TPA. However, i.p. injections of Put (0.1–5.0 mg) alone did not affect epidermal OrnDCase nor did they provide for papilloma formation (data not shown). These results were very reproducible as we have previously discussed (10, 29, 35). Put levels were abnormally high in the papillomas when compared with skin tissue (Table 2). It is important to note that at 24 hr after one TPA application Put values returned to near control levels (Table 1 and Fig. 1). O'Brien had previously observed aberrant OrnDCase activity in papilloma and carcinoma tissue compared with control skin values (33).

Because Put enhanced TPA promotion and because levels in papillomas were abnormally high compared with those in surrounding skin tissue, we postulated that specific inhibition of Put biosynthesis by CHF_2-Orn should decrease tumorigenesis in mouse skin. CHF_2-Orn (2.0 mg) eliminated the TPA-increased OrnDCase activity and retained the epidermal Put level at or near control values through 3 days after one TPA application (Table 3 and Fig. 1). CHF_2-Orn also decreased the TPA-enhanced Spd accumulation by >35% at 24 hr but had little effect on Spm levels. However, in initiated epidermis [100 nmol of $(CH_3)_2BA$], we found that CHF_2-Orn (2.0 mg) lost full effectiveness after four successive biweekly applications; between

Table 1. Comparative effects of TPA, mezerein, and EPP on epidermal polyamine biosynthesis

Agent	Dose, μg	Time, hr	OrnDCase activity, nmol CO_2/mg protein/hr	Polyamine level, nmol/mg protein		
				Put	Spd	Spm
Acetone		0	0.1	0.9	5.2	2.8
TPA	2	6	11.8	1.7	6.4	2.0
		9		2.9	7.6	2.4
		12		2.7	9.8	2.4
		24		0.7	10.3	3.0
		48		0.7	9.3	2.6
Mezerein	2	6	17.5	2.9	7.3	2.9
		9		2.7	8.5	2.3
		12		4.3	9.9	2.0
		24		1.3	10.4	2.4
		48		1.0	9.8	2.6
EPP	3,000	6	2.4	1.1	5.7	2.8
		9		1.0	5.8	3.1
		12		0.9	6.1	2.8
		24		1.2	6.5	3.3
		48		0.8	4.1	2.3

TPA, mezerein, or EPP was applied once topically in 0.2 ml of acetone to dorsal skin. At times indicated after treatment, groups of 3–5 mice were sacrificed for OrnDCase activity assessment or for polyamine analysis or both. Variation for OrnDCase was <±15%. Range for polyamines was: Put and Spd, <±10%; Spm ± 15%.

Table 2. Polyamine levels in "autonomous" papillomas and in "uninvolved" skin after 16 wk of treatment

Treatment	Papillomas per mouse	Polyamine level, nmol/mg protein		
		Put	Spd	Spm
TPA (1 μg)	11.7	5.3 (1.5)	11.1 (7.7)	1.8 (2.5)
TPA (1 μg) + Put·2 HCl	19.3	8.2 (1.1)	9.8 (5.8)	1.7 (2.1)

Mice, 30 per group, were initiated with 100 nmol of $(CH_3)_2BA$ and the following week were given TPA (1 μg) with or without i.p. doses of Put (0.25 mg) in P_i/NaCl biweekly for 16 wk. Tissues for polyamines were recovered 24 hr after the last treatment. Values for uninvolved skin are given in parentheses. Triplicate analyses were performed; average values are given and the range was within ±14%. Papilloma variation within each group was <20%.

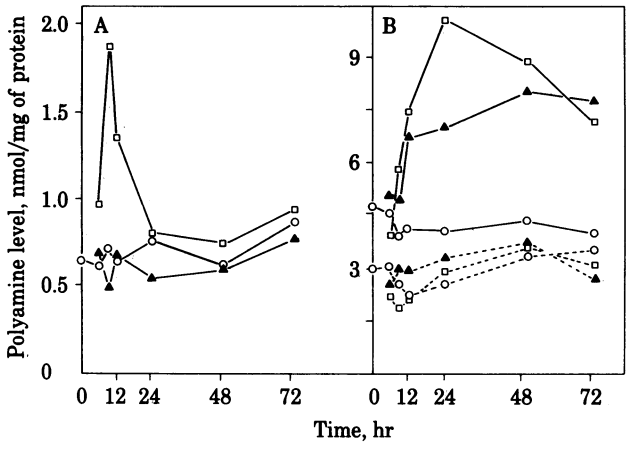

FIG. 1. Polyamine levels in epidermis of 8-wk-old female SENCAR mouse after one treatment with TPA (1 μg) in acetone and, when indicated, CHF_2-Orn (2 mg, i.p. in 0.1 ml of P_i/NaCl). Groups of four or five mice were killed at indicated times. Values are averages of triplicate determinations from at least two trials and were within ±10%. ○, Acetone or P_i/NaCl control (or both); □, TPA treatment; ▲, TPA + CHF_2-Orn. (*A*) Put; (*B*) Spd (——) and Spm (---) levels.

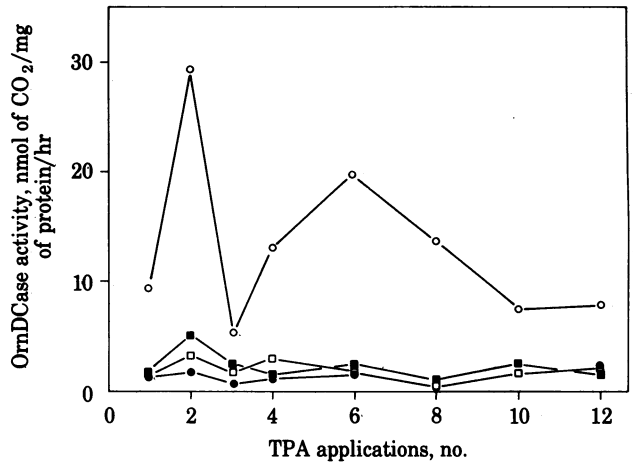

FIG. 2. Effect of CHF_2-Orn on epidermal OrnDCase activity after multiple applications of TPA. ○, TPA (2 μg) applied biweekly for 6 wk; ●, TPA (2 μg) + CHF_2-Orn (2%) in drinking water; □, CHF_2-Orn (20 mg), i.p. twice weekly with TPA (2 μg); ■, CHF_2-Orn (5 mg), i.p. twice weekly with TPA (2 μg). OrnDCase activity was determined 6 hr after indicated TPA treatments. Three or four mice were used per group per time point. Triplicate analyses yielded a range of ±15% from the mean. Control OrnDCase activity was 0.10 ± 0.07 nmol of CO_2 per mg of protein per hr. Mice were initiated with 10 nmol of $(CH_3)_2BA$ 1 wk prior to commencing TPA treatment (see Tables 4 and 5).

12 and 72 hr after the fourth treatment, both TPA-treated mice and TPA- and CHF_2-Orn-treated mice had relatively similar epidermal Put levels, which were 1.3–1.9 times control values (35). Others had previously reported that a continuously high level of CHF_2-Orn was necessary for maximal effect (19, 22, 23, 32).

Thus, we employed higher dose levels of CHF_2-Orn and assessed the effects on OrnDCase and polyamine levels in initiated–promoted skin over a 6-wk observation period, as shown in Fig. 2 and Table 4. A peak of >300-fold induction of OrnDCase activity occurred after the second application of TPA; TPA-enhanced epidermal OrnDCase activity reached a secondary plateau between 3 and 4 wk. A replicate experiment provided the same pattern. Over the entire observation period CHF_2-Orn, given i.p. at 5 or 20 mg twice weekly with TPA or administered at a level of 2% (wt/vol) continuously in drinking water, effectively abolished the TPA-induced OrnDCase activity (Fig. 2). At 6 wk, the Put level in the TPA-treated group was >2-fold higher than that of controls, the Spd level was 135% of control, but the Spm level remained constant (Table 4). These early changes in time and magnitude of Put fluctuation are similar to the results obtained during complete carcinogenesis (36, 37). CHF_2-Orn effectively, and in a dose-dependent manner, inhibited Put accumulation (Table 4). At higher levels of CHF_2-Orn, an inhibition of Spd accumulation along with elevated Spm levels was observed. These results are in agreement with previous reports (19, 22, 32).

The effects of CHF_2-Orn on TPA promotion are depicted in Fig. 3 and Table 5. CHF_2-Orn in drinking water decreased papilloma formation by 25% and given i.p. at 20 mg per dose it reduced tumor yield by 18–20%, whereas at the 5-mg level it had essentially no effect. These results did not fully conform with our previous results using a slightly different protocol (10, 29, †) nor did they demonstrate the expected inhibitory effectiveness of CHF_2-Orn based on its ability to inhibit OrnDCase activity and polyamine levels; however, we did observe a dramatic difference in the size of the papillomas obtained at 18 wk (Fig. 3 and Table 5). The average size (±SD) of the papillomas in animals that received CHF_2-Orn in drinking water was 2.5 ± 0.5 mm. Furthermore, all papilloma lesions were closely grouped in size range and were of very similar diameter. The group that was treated with TPA only yielded a pattern of wide diversity in papilloma size [average diameter (±SD), 5.5 ± 2.0 mm]. Also, it was evident that CHF_2-Orn provided a dose–size response relationship (Fig. 3 and Table 5). Verma *et al.* (15) had similarly observed a decreased papilloma size after treatment with retinoids, which inhibit OrnDCase and Put accumulation

Table 3. Effect of CHF_2-Orn on TPA-induced epidermal OrnDCase activity

Experiment	Modifier	CHF_2-Orn dose, mg	OrnDCase activity, nmol CO_2/mg protein/hr
1	Acetone	—	0.02
	TPA	0.001	3.93
	TPA ± CHF_2-Orn	2.0	0.11
	TPA ± CHF_2-Orn	0.5	0.65
2	Acetone	—	0.08
	TPA	0.001	4.57
	TPA ± CHF_2-Orn	2.0	0.06
	TPA ± CHF_2-Orn	0.5	0.42

Groups of four mice each were treated once topically with TPA with or without coincident i.p. injections of CHF_2-Orn. Epidermal OrnDCase activity was assayed 5.5–6 hr later. Triplicate evaluations per group were performed; variation was ± 15%. Data from two representative experiments are shown.

Table 4. Effect of CHF_2-Orn on epidermal polyamine levels after 6 wk of TPA application

Treatment	Polyamine level, nmol/mg DNA		
	Put	Spd	Spm
TPA (2 μg)	16.3	79.6	34.2
TPA (2 μg) + CHF_2-Orn (2% in H_2O)	4.1	65.3	46.3
TPA (2 μg) + CHF_2-Orn (20 mg)	6.6	65.3	40.5
TPA (2 μg) + CHF_2-Orn (5 mg)	8.1	75.7	35.0
Control (acetone)	7.4	59.6	34.6

Groups of four mice each were taken from the tumor experiment groups (Table 5). Mice were sacrificed 24 hr after 6 wk of treatment. Values are average of triplicate analyses with variation <10%.

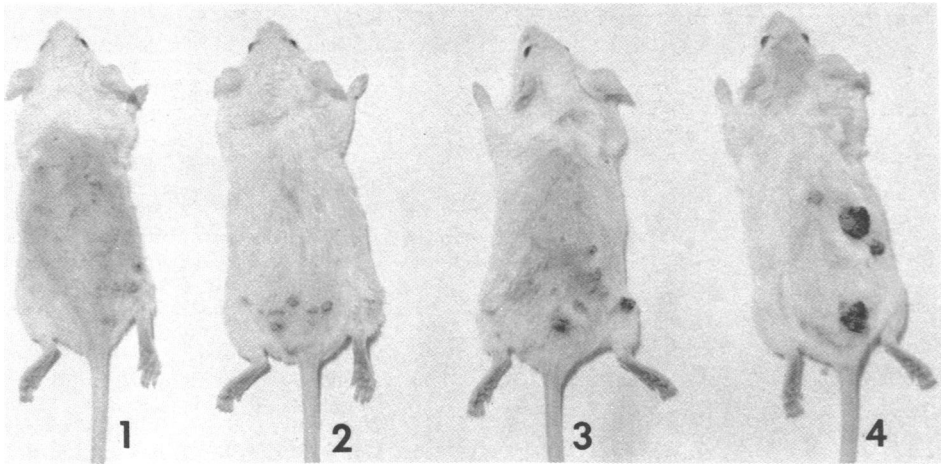

FIG. 3. Representative mice from each group in tumorigenesis experiment described in Table 5. Groups: 1, TPA + CHF_2-Orn (2%) in H_2O; 2, TPA + CHF_2-Orn (20 mg); 3, TPA + CHF_2-Orn (5 mg); 4, TPA treatment only.

in proportion to their inhibition of tumor promotion. Another striking observation was that hair regrowth was severely retarded by CHF_2-Orn throughout the 18-wk experimental period and this phenomenon also was dose related. At the termination of the experiment, gross necropsy examination revealed no striking abnormalities (unpublished data). Similarly, histological sections of the skin showed no significant differences in the number of dark basal keratinocytes between the animals treated with TPA only and the mice receiving CHF_2-Orn (unpublished data).

Results obtained for OrnDCase and tissue polyamine levels are presented in Table 5. Two facts are strikingly evident: (*i*) OrnDCase activity and Put levels are considerably elevated in papillomas when compared with surrounding skin tissue, and (*ii*) CHF_2-Orn in drinking water and i.p. at 20 mg per dose effectively lowered Put accumulation in skin and papillomas, but the OrnDCase activity and Put levels were significantly greater in the papillomas than in the surrounding skin tissue of the CHF_2-Orn-treated groups and were also elevated over the control nontreated skin values. However, the *S*-adenosylmethionine decarboxylase activity alterations in skin or papilloma tissue were minimal; TPA-treated group values ranged between 0.12 and 0.17 nmol of CO_2 per mg of protein per 30 min, whereas enzyme activity in the CHF_2-Orn-treated groups ranged from 0.10 to 0.28 nmol of CO_2 per mg of protein per 30 min.

Because polyamine burden—particularly Put levels—correlated with papilloma size/growth rate (Table 5 and Fig. 3) and because mezerein induced changes in polyamine biosynthesis similar to those induced by TPA (Table 1), we utilized CHF_2-Orn in a stage-specific promotion protocol (3, 5). As shown in Fig. 4, CHF_2-Orn given with TPA did not inhibit papilloma formation. However, CHF_2-Orn given in the second stage (mezerein treatment) led to a 65–70% reduction in the number of papillomas per mouse, a significant reduction in their size, and a 40% reduction in tumor incidence. A replicate experiment provided similar results. These results dramatically support our earlier hypothesis (3–5) that (*i*) TPA, a complete promoting agent, brings about a required preliminary conversion in cellular function (dark basal keratinocytes) that the weak stage II

Table 5. Effect of CHF_2-Orn on OrnDCase activity, polyamine levels in papillomas and skin tissue, and tumor promotion by TPA

Treatment	Tumor data			OrnDCase activity, nmol CO_2/mg protein/hr		Polyamine level, nmol/mg DNA					
						Put		Spd		Spm	
	% of mice with tumors	Papillomas, no./mouse	Size, mm*	Skin	Pap.	Skin	Pap.	Skin	Pap.	Skin	Pap.
TPA (2 μg)	92	4.5	5.5 ± 2.0	1.8	12.3	11.5	152	83	201	35	48
TPA (2 μg) + CHF_2-Orn (2% in H_2O)	81	3.4	2.5 ± 0.5	0.6	3.3	4.3	17	74	176†	28	65
TPA (2 μg) + CHF_2-Orn (20 mg, i.p.)	84	3.7	3.0 ± 0.5	0.4	3.8	6.9	12	92	242	39	69
TPA (2 μg) + CHF_2-Orn (5 mg, i.p.)	77	4.4	4.0 ± 1.0	1.2	4.3	8.0	109	106‡	224	36	51
Control (acetone or P_i/NaCl, i.p.)	—	—	—	0.1	—	8.5	—	70	—	36	—

Groups of 33 female SENCAR mice were initiated with 10 nmol of $(CH_3)_2BA$ at 7 wk of age; promotion began at 8 wk and was continued for 18 wk. CHF_2-Orn in drinking water (1.5–2.0% wt/vol) was given continuously and the average intake (±SD) was 8.53 ± 0.89 ml per mouse per day (4.7 ± 0.5 g/kg/day). The group treated with TPA only drank (mean ± SD) 8.18 ± 0.84 ml per mouse per day. Average weight gains per mouse were: TPA only, 5.28 g; TPA + CHF_2-Orn (5 mg), 4.31 g; TPA + CHF_2-Orn (20 mg), 4.69 g; TPA + CHF_2-Orn (2% in H_2O), 4.45 g. Maximal SD for tumor yield was 16% for the groups. Size of papillomas was determined by examination of at least six mice per group. OrnDCase activity was determined 6 hr after the last TPA application. Polyamine levels were determined in the specified tissues at 24 hr after TPA application. Variation was <10% from the mean in triplicate trials except where noted. Skin, surrounding skin; pap., papilloma.

* Average ± range.

† Range ≤12%.

‡ Range ≤17%.

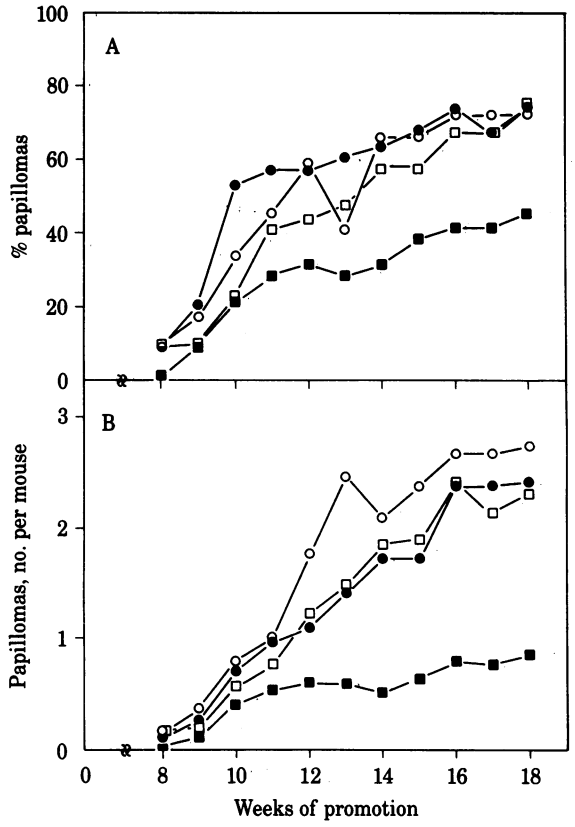

FIG. 4. Effects of CHF_2-Orn on two-stage (TPA–mezerein) promotion tumorigenesis. Mice were initiated with 10 nmol of $(CH_3)_2BA$ and promoted with 2 μg of TPA (four applications); this was followed by mezerein (2 μg) applications for 16 wk. CHF_2-Orn, 20 mg i.p., was given either with TPA or with mezerein at biweekly intervals. ●, TPA + CHF_2-Orn; ■, mezerein + CHF_2-Orn; ○, TPA + P_i/NaCl; □, mezerein + P_i/NaCl. The maximal SD for the groups was 18%. (*A*) Papilloma formation; (*B*) tumor incidence.

promoter mezerein is not capable of providing. The polyamines are induced by TPA, but do not fulfill *per se* the essential function of TPA (i.e., Put alone is not a promoter nor does it alter dark cell induction); and (*ii*) polyamine induction by mezerein provides a critical function in stage II promotion which we demonstrate is specifically inhibited by CHF_2-Orn. Our data obtained in mouse skin, taken together with recent reports by others (32, 38–41), strongly indicate the potential utility of CHF_2-Orn in chemotherapeutic as well as chemopreventative regimens.

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