[CANCER RESEARCH 47, 1775–1779, April 1, 1987]

12-*O*-Tetradecanoylphorbol-13-acetate-dependent Induction of Xanthine Dehydrogenase and Conversion to Xanthine Oxidase in Murine Epidermis[1]

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ABSTRACT

Both xanthine dehydrogenase (XD) and xanthine oxidase (XO) catalyze the conversion of hypoxanthine to xanthine, and xanthine to uric acid. Topical application of a promoting dose of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to the dorsal skin of female SENCAR mice resulted in a 3.0–3.5-fold elevation of epidermal XO specific activity. Epidermal XO specific activity was maximally elevated 48–96 h after TPA treatment, and required 11 days to return to control levels. Although TPA increased the XO/(XD + XO) ratio from 0.45 to 0.7, the conversion of preexisting XD to XO could not solely account for the TPA-dependent elevation in XO specific activity since control XD plus XO activity was less than just the XO activity in TPA-treated epidermis. Topical application of cycloheximide simultaneously with, or 12 h after, TPA treatment inhibited the TPA-dependent increases in the XO/(XD + XO) ratio and XO specific activities. Collectively, these results suggest that the increased XO activity detected following TPA treatment is the consequence of TPA-induced XD synthesis, and a conversion of existing and newly synthesized XD to XO. In addition, the *in vivo* promoting activities of analogues of TPA could be correlated with their abilities to elevate XO activity (TPA > phorbol-12,13-dibenzoate ≫ 4-*O*-methyl-TPA = phorbol).

INTRODUCTION

Skin tumors can be experimentally induced by the sequential application of a subthreshold dose of carcinogen (initiation phase) if followed by repetitive treatment with a noncarcinogenic promoter (promotion phase) (1). Although the critical molecular and biochemical events intrinsic to the processes of promotion are poorly understood, a substantial data base has accumulated implicating a role for the reactive oxygen species superoxide and its reduction products, hydrogen peroxide and hydroxyl radical, in skin tumor promotion (reviewed in Refs. 2–4).

The sources of reactive oxygen species relevant to the processes of promotion in murine skin are unknown. Although *in vitro* treatment of polymorphonuclear leukocytes and macrophages with TPA[4] results in the generation of reactive oxygen species (5, 6), and these cell types are known to infiltrate the dermis following TPA treatment of the epidermis (7), their contribution to TPA-dependent promotion is speculative. Similarly, recent studies by Fischer and Adams (8) suggest that TPA can stimulate the production of superoxide by keratinocytes. However, the source of the superoxide in their studies is unknown.

XD and XO are purine catabolism pathway enzymes responsible for the conversion of hypoxanthine to xanthine, and xanthine to uric acid. As a dehydrogenase XD uses NAD^+ as an oxidant, whereas XO uses O_2 as an oxidant resulting in the production of superoxide. Della Corte and Stirpe (9, 10) originally demonstrated that rat liver XD could be converted to XO by a variety of *in vitro* treatments including limited proteolysis, storage at −20°C, heat, and treatment with sulfhydryl modifying reagents. Recently it was demonstrated that the XD to XO conversion is not solely an *in vitro* phenomenon. Interferons (11) and ethanol (12) have been reported to affect the *in vivo* conversion of murine liver and rat heart XD to XO, respectively, Furthermore, the hepatic and cardiac toxicity associated with *in vivo* interferon treatment and alcohol administration could be directly related to elevated XO activity (11, 13), and in the case of the interferon experiments to the resultant production of superoxide (11).

In the current study we demonstrate that topical application of the tumor promoter TPA to SENCAR mouse skin results in an elevation of epidermal XO activity. This elevation is due to a TPA-dependent increase in XD synthesis, and a conversion of XD to XO. Furthermore, the *in vivo* promoting capacity of TPA analogues correlate with their abilities to elevate XO activity.

MATERIALS AND METHODS

Chemicals. Hypoxanthine, xanthine, uric acid, and cycloheximide were purchased from the Sigma Chemical Company. Radiolabeled [8-^{14}C]hypoxanthine (50.9 mCi/mmol) was purchased from Amersham. Radiolabeled L-[^{3}H]leucine (58 Ci/mmol) was purchased from ICN. Cellulose thin-layer chromatography plates were obtained from the Eastman Kodak Company. TPA, phorbol-12,13-dibenzoate, 4-*O*-methyl-TPA and mezerein were purchased from Chemicals for Cancer Research, Inc., Eden Prairie, MN.

Animal Treatment Protocols. The dorsal skins of female SENCAR mice (6–7 weeks old; Research Biogenics, Inc., Bastrop, TX) were shaved 3–7 days before treatment with chemicals. Animals in hair regrowth were not used. All chemicals were dissolved in acetone and applied topically in a volume of 0.2 ml. Control animals were treated with only acetone.

Tissue Preparation. Two procedures were used for the preparation of murine epidermal homogenates. In protocol 1 mice were killed by cervical dislocation and Nair depilating agent (Carter-Wallace, Inc.) was applied to the shaved area of the back. After 2 min the dorsal skin was first washed with cold water and then excised with scissors. The excised skin pelt was then sequentially immersed in an ice-water bath (5 min), 55°C water bath (0.5 min), and ice-water bath (5 min). Following the third immersion the skin pelt was blotted dry and placed dermis side down on a glass plate and the epidermis was scraped off with a razor blade. In protocol 2 the Nair, ice bath, and 55°C treatments were omitted and the epithelial side of the skin pelt was scraped directly. Epithelial scrapings from either protocol were suspended in 0.5 ml of 50 mM $NaKPO_4$, pH 7.0, 0.1 mM EDTA and homogenized with a Brinkmann polytron. The homogenate was centrifuged for 15 min at 13,500 × *g* and the supernatant fluid was recentrifuged for 5 min at 13,500 × *g*. The resulting supernatant fluid was used either immediately or divided into several fractions and stored at −20°C.

[^{3}H]Leucine Incorporation into Protein. Mice were killed 1 h after i.p. injection of 20 μCi L-[^{3}H]leucine diluted in 0.2 ml 0.15 M NaCl.

Received 9/22/86; revised 12/17/86; accepted 12/30/86.

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[1] This project was supported by National Cancer Institute Grants CA-40823 and CA-34469, Department of Health and Human Services, and by American Cancer Institute Grant IFN-16.

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[4] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDBz, phorbol-12,13-dibenzoate; XD or D, xanthine dehydrogenase; XO, total xanthine oxidase; O, reversible xanthine oxidase; O′, irreversible xanthine oxidase; DTT, dithiothreitol; PMN, polymorphonuclear leukocyte.

Epidermal homogenates were prepared by tissue preparation protocol 1 minus the heat treatment. Protein in the 13,500 × *g* supernatant fluids was precipitated by addition of an equal volume of 12% TCA and incubation at 4°C for 2 h. The precipitate was pelleted by centrifugation, and washed twice with 5% TCA. The precipitate was suspended in 0.2 N NaOH and solubilized by overnight incubation at 37°C. The resulting supernatant fluid was used for protein and radioactivity analyses. Protein was estimated by the method of Lowry *et al.* (14) using bovine serum albumin as the standard.

Xanthine Dehydrogenase/Oxidase Assay. Xanthine dehydrogenase/oxidases were assayed by a modification of the procedure of Mousson *et al.* (15). Dehydrogenase activity is distinguished from total oxidase activity by using NAD^+ as the oxidant. There are two forms of XO activity termed reversible (O) and irreversible (O′) xanthine oxidase (16). Reversible O can be converted to XD by inclusion of a sulfhydryl-reducing agent such as DTT. Consequently, irreversible O′ activity is determined by inclusion of DTT in the oxidase cocktail assay, and reversible O activity is calculated as the difference between total XO activity and O′ activity.

Xanthine dehydrogenase plus total XO activities were assayed in a reaction mixture (final volume 40 μl) containing enzyme preparation, 40 nmol NAD^+, 2 μmol Tris-HCl (pH 9.0), and 10 μl of [8-^{14}C] hypoxanthine (9 μCi/ml, 50–53 mCi/mmol). Total XO activity was assayed similarly but in the absence of NAD^+. Irreversible xanthine oxidase was assayed in the XO cocktail supplemented with 0.4 μmol DTT. Reactions were initiated by the addition of enzyme preparation and incubated for 5–10 min at 37°C. Protein content of the enzyme preparation was adjusted to insure that product formation was directly proportional to protein concentration and length of assay. Reactions were terminated by the addition of 20 μl of 1 M perchloric acid and centrifuged for 2 min at 13,500 × *g*. Five μl of the supernatant fluid were spotted on cellulose thin-layer chromatography plates prespotted with 2.5 μl of 10^{-2} M solution of unlabeled hypoxanthine, xanthine, and uric acid. Thin-layer chromatography plates were developed with a $CH_3(CH_2)_2CH_2OH/CH_3OH/H_2O/NH_4OH(25\%)$ (60/20/20/1) mixture and reference metabolites were located with a UV light. Areas of the TLC plates corresponding to xanthine and uric acid were excised and analyzed by liquid scintillation counting. Although xanthine and uric acid are resolved by the chromatography procedure they were counted collectively. A reaction mixture containing no enzyme preparation was processed in parallel as a control and subtracted from the experimental values. Specific activity is expressed as nmoles of hypoxanthine oxidized to xanthine plus uric acid/min/mg protein. Protein content was determined with the Bio-Rad protein assay reagent using bovine serum albumin as a standard.

Statistical analyses were performed using Student's *t* test, two-tailed, with pooled variances.

RESULTS

Effects of Tissue Preparation and Storage on XO and XD Activities. A depilatory agent and a temperature shock protocol employing a 30-s immersion into 55°C water are commonly used for the preparation of murine skin epidermal extracts (17). However, prolonged incubation at 37°C and exposure to agents that modify or oxidize sulfhydryls have been reported to convert rat hepatic XD into XO (9, 10, 16). We have found that use of the depilating agent Nair and immersion of skin into 55°C water for 30 s, either employed singularly or in combination, does not alter the conversion of XD to XO (Table 1). This is evident from the finding that the treatments have no effect on the epidermal XO/(XD + XO) ratio. However, a single cycle of freeze-thawing resulted in an increased XO/(XD + XO) ratio (Table 1) and is indicative of a conversion of XD to XO. Furthermore, incubation of epidermal extracts for longer than 15 min at 37°C also resulted in a conversion of XD into XO (Table 1). Consequently, for those studies in which the ratio of XO/(XD + XO) activity was critical, activity analyses were performed immediately after tissue homogenization.

Table 1 *Effects of tissue preparation procedure, heat, and freezing on conversion of XD to XO*

Epidermal homogenates were prepared from skin pelts of mice that had been treated with the depilating agent Nair and then subjected to a temperature shock, or Nair or temperature shock individually as described in "Materials and Methods." Alternately epidermal homogenates were prepared from non-Nair and nonheat shocked skin pelts. XO and XD activities were assayed immediately after tissue preparation or storage for 48 h at −20°C. Homogenates were preincubated for 0–60 min prior to being assayed at 37°C for 5 min.

Tissue treatment	Incubation at 37°C (min)	XO / (XD + XO) (%) Fresh	XO / (XD + XO) (%) Frozen
None	5	40.48 ± 3.53	54.00 ± 1.41[a, b]
55°C, Nair	5	40.07 ± 4.19	63.11 ± 3.76[a]
55°C	5	40.64 ± 2.20	66.18 ± 1.20[a]
Nair	5	35.30 ± 0.32	66.00 ± 2.1[a]
None	5	37.48 ± 1.71	
None	10	39.13 ± 2.12	
None	15	37.20 ± 0.77	
None	20	42.87 ± 0.31	
None	40	42.49 ± 1.46	
None	65	51.80 ± 0.82	

[a] Statistically different from values obtained with fresh extract, $P < 0.05$.
[b] *Values,* mean ±SE.

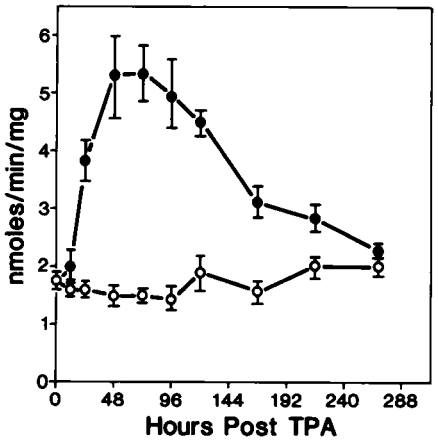

Fig. 1. Kinetics of TPA-dependent elevation of XO activity in murine epidermis. Mice were topically treated with either acetone (○) or 2 μg TPA (●) and sacrificed at various times after treatment. Data points represent the mean ± SE of a minimum of six animals.

TPA-dependent Elevation of Xanthine Dehydrogenase/Oxidase. Topical application of a promoting dose of TPA (2 μg) to the dorsal skin of SENCAR mice resulted in a 3–3.5-fold elevation of epidermal XO activity (Fig. 1). Total XO specific activity was significantly increased within 24 h of TPA treatment and reached a maximum within 48 h. This maximum was sustained for 48 h. Xanthine oxidase specific activity did not return to the control level until 11 days after TPA treatment.

The elevated XO specific activities following TPA treatment could be the consequence of either the conversion of existing XD to XO, or *de novo* synthesis of XD coupled with conversion of existing and newly synthesized XD to XO. An additional complication is that there are two forms of XO. The first form, termed reversible xanthine oxidase (O) can be converted to XD by incubation with sulfhydryl reducing agents such as DTT. The second form of XO, termed irreversible xanthine oxidase (O′), cannot be converted into XD. The three activities (D, O, O′) can be distinguished by using different combinations of oxidants and the inclusion of DTT (Table 2). Within 48 h of TPA treatment irreversible O′ and XD plus XO specific activities were both increased approximately 2-fold by TPA treatment. Total oxidase specific activity was increased approximately 3-fold. The increase in total oxidase activity was due to

Table 2 *Assay conditions for differential analyses of xanthine dehydrogenase/oxidase activities*

Epidermal homogenates were prepared from the dorsal skins of mice 48 h after treatment with either acetone or TPA and assayed as described in "Materials and Methods."

Oxidant in assay	DTT	Activity measured	nmol/min/mg	
			Acetone	TPA
O_2	–	O + O′	1.74 ± 0.19	5.80 ± 0.33[a, b]
O_2 + NAD^+	–	D + O + O′	4.08 ± 0.42	8.34 ± 0.37[a]
O_2	+	O′	0.94 ± 0.08	2.17 ± 0.08[a]
O_2 + NAD^+	+	D + O + O′	4.39 ± 0.41	9.24 ± 0.43[a]

[a] Significantly different from acetone-treated animals, $P < 0.001$.
[b] *Values,* mean ±SE of duplicate determinations from two experiments each containing four animals per treatment.

Table 3 *Composition of XD and XO activities in murine epidermis following TPA treatment*

Epidermal homogenates were prepared from the dorsal skins of mice 48 h posttreatment. Data are from Table 2.

Treatment	% of total activity		
	D / D + O + O′	O + O′ / D + O + O′	O′ / O + O′
Acetone	54.5 ± 2.7[a]	45.5 ± 2.7	51.9 ± 1.8
TPA	30.0 ± 3.8[b]	70.0 ± 3.8[b]	38.2 ± 2.8[c]

[a] *Values,* mean ±SE obtained from duplicate determinations from two experiments, each containing four animals per treatment.
[b] Significantly different from acetone-treated animals, $P < 0.001$.
[c] Significantly different from acetone-treated animals, $P < 0.01$.

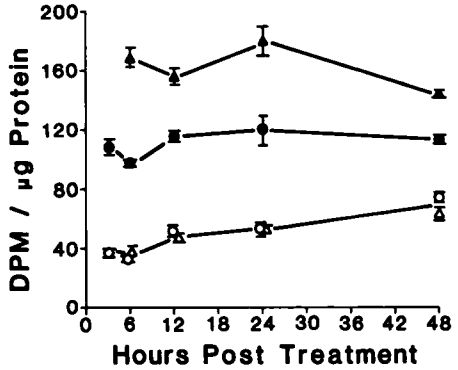

Fig. 2. Kinetics of cycloheximide inhibition of leucine incorporation into cytoplasmic epidermal polypeptides. Mice were pulse labeled with L-[^{3}H]leucine for 1 h prior to sacrifice. Epidermal homogenates were prepared from the dorsal skins of mice at various times after topical treatment with acetone (●), acetone plus cycloheximide (○), TPA (▲), and TPA plus cycloheximide (△). Cycloheximide [2 mg in 0.2 ml acetone (32)] was simultaneously applied with 2 µg TPA. Data are the mean ± SE of quadruplicate analyses of two animals per treatment.

the conversion of XD to the oxidase. Approximately 70% of the total XD + XO activity in TPA-treated mice was XO activity, whereas only 45% of the total activity was XO in control animals (Table 3). The findings that XO activity of TPA-treated epidermis was greater than the total XD + XO activity of control epidermis, but XD specific activity was unaffected (Table 2), suggested that TPA induces the synthesis of XD and both existing and newly synthesized XD were being converted to the oxidase in TPA-treated mice. This hypothesis is supported by studies with the protein synthesis inhibitor cycloheximide (Fig. 2, Table 4). Topical treatment with TPA enhanced epidermal protein synthesis (Fig. 2). The magnitude of the TPA enhancement at 24 and 48 h is considerably less than the 4-fold increase reported by Raick (18), and may be the consequence of differences in procedure. Whereas Raick measured leucine incorporation into whole epidermis, we have only measured leucine incorporation into cytoplasmic polypeptides, which may be more appropriate for our studies because XD/XO are cytoplasmic enzymes. Topical application of cycloheximide resulted in a prolonged suppression of [^{3}H]leucine incorporation into cytoplasmic epidermal proteins of both control and TPA-treated mice (Fig. 2). Similarly, simultaneous treatment of mice with TPA and cycloheximide, or with cycloheximide 12-h post-TPA treatment, essentially eliminated any TPA-induced increase in XO specific activity, and reduced the XO/(XD + XO) ratio to near control levels (Table 4).

Table 4 *Effects of cycloheximide treatment on TPA-dependent XD/XO activities in murine epidermis*

Epidermal homogenates were prepared from the dorsal skins of mice 48 h after treatment with acetone or TPA. Cycloheximide (CHX) was applied topically (2 mg in 0.2 ml acetone) either simultaneously (0 time) or 12 h after TPA or acetone treatment.

Treatment	nmol/min/mg		
	XO	XO + XD	XO / XO + XD (%)
Acetone	1.74 ± 0.19[a]	4.08 ± 0.42	45.5 ± 2.7
Acetone + CHX (0)	1.53 ± 0.01	3.01 ± 0.32	52.2 ± 5.8
Acetone + CHX (+12)	1.31 ± 0.04	2.42 ± 0.05	54.2 ± 2.9
TPA	5.80 ± 0.33	8.34 ± 0.38	70.0 ± 3.8
TPA + CHX (0)	1.84 ± 0.13[b]	3.29 ± 0.22[b]	57.2 ± 7.2
TPA + CHX (+12)	2.35 ± 0.22[b]	4.62 ± 0.36[b]	51.4 ± 4.9[c]

[a] *Values,* mean ±SE of duplicate determinations of three animals.
[b] Significantly different from TPA-treated animals, $P < 0.001$.
[c] Significantly different from TPA-treated animals, $P < 0.05$.

Table 5 *Elevation of XO in murine epidermis by TPA and its analogues*

Epidermal homogenates were prepared from the dorsal skins of mice 48 h posttreatment.

Treatment	Dose (µg)	% of control XO activity
Acetone		100.0
TPA	0.5	167.0 ± 9.6[a, b]
	1.0	188.7 ± 8.4[b]
	2.0	291.6 ± 11.7[b]
Phorbol	2.0	98.3 ± 8.1
	20.0	94.3 ± 6.0
4-*O*-MeTPA[c]	2.0	120.7 ± 5.1
	20.0	100.7 ± 4.6
PDBz	2.0	148.9 ± 7.6[b]
	10.0	178.9 ± 7.0[b]
Mezerein	1.0	259.8 ± 22.7[b]
	2.0	292.3 ± 41.0[b]
	4.0	244.8 ± 36.1[b]

[a] *Values,* mean ±SE obtained from duplicate determinations of samples obtained from a minimum of four mice.
[b] Significantly different from acetone-treated animals, $P < 0.01$.
[c] 4-*O*-MeTPA, 4-*O*-methyl-TPA.

Survey of Phorbol Analogues. The relative *in vivo*-promoting activities of TPA and its analogues could be correlated with their abilities to elevate XO activities (Table 5). A dose response increase in XO specific activity was measured for topical applications of 0.5–2 µg of TPA (Table 5). In SENCAR mice 0.5 µg of TPA is a very weak promoting dose whereas 2 µg of TPA is the standard and optimum dose (19). The weak tumor promoter phorbol-12,13-dibenzoate, at a dose of 10 µg/application, resulted in a significant elevation of XO which was comparable to the value obtained with 0.5 or 1 µg of TPA. The nontumor-promoting agent phorbol, and the first-stage promoter 4-*O*-methyl-TPA had no effect on XO activity. Mezerein, a potent second-stage promoter in SENCAR mice (1), on a molar basis was as effective as TPA in elevating XO activity.

DISCUSSION

Topical application of a promoting dose of TPA resulted in a 3–3.5-fold increase in epidermal XO specific activity. This increase was due to a TPA-dependent stimulation of XD synthesis, and the conversion of XD to XO. Two types of data collectively suggest that both preexisting and newly synthesized XD are converted to XO. First, topical treatment with cyclo-

heximide suppressed the TPA-dependent stimulation of epidermal protein synthesis, and elevations in XO activity and the XO/(XD + XO) ratio. Second, since the total XO + XD specific activity of control animals is significantly less than just the XO activity of TPA-treated animals, and XD and XO have a very similar K_m and V_{max} for substrate (20), at least a portion of the XO activity in TPA-treated animals must be contributed by the conversion of newly synthesized XD to XO. It should be further emphasized that while a variety of agents and handling and storage practices can affect the artifactual conversion of XD to XO, we have employed tissue preparation, and storage and assay protocols that minimize the XD to XO conversion. Furthermore, since total extractable epidermal protein increases dramatically following TPA treatment of SENCAR mice (21) the measured increases in XO specific activity represent a true increase in overall XO activity and protein in the epidermis.

Superoxide and its reduction products (hydrogen peroxide, hydroxyl radical) are genotoxic-reactive oxygen species (2–5). Whereas XD cannot use molecular oxygen as an oxidant, XO can, which results in the production of superoxide. Elevated XO activity, mediated by the conversion of XD to XO is associated with several pathological conditions including intestinal ischemia (22), ethanol-associated myocardial (12, 13) and hepatic toxicity (12), and interferon-induced hepatic lipoperoxidation (23) and loss of hepatic mixed-function oxidase activities (11, 23). The findings that the XD/XO inhibitor allopurinol can eliminate or lessen the severity of tissue injury in the aforementioned examples (11, 12) suggests that XO is a causative factor of the cytotoxicity. Furthermore, the ability of antioxidants to inhibit the toxic effects of ethanol (24) and interferons (11) suggest that it is the superoxide produced by XO, or the reduction products of superoxide that are responsible for the cytotoxicity.

A substantial data base implicates a role for reactive oxygen species in tumor promotion (2–4). Included in this data base are the following observations: (*a*) antioxidants are generally antipromoting agents (25); *b*) topical application of TPA to murine dorsal skin reduces the specific activities of epidermal superoxide dismutase and catalase, enzymes involved in the detoxification of reactive oxygen species (21, 26); (*c*) the superoxide dismutase biomimetic copper complex Cu(II)(3,5-diisopropylsalicylate)$_2$ can inhibit TPA-dependent promotion in CD-1 mice (27, 28); and (*d*) *in vitro* reactive oxygen generating systems can mimic the effects of tumor promoters in some systems (2–4, 29). Relevant to this final category and our studies is a report by Zimmerman and Cerutti (29) that employed xanthine-xanthine oxidase as a superoxide generating system. They demonstrated that extracellularly produced superoxide could promote the transformation of initiated mouse embryo C3H/10T1/2 fibroblasts in culture. Similarly, it has recently been demonstrated by Fischer *et al.* (30) that supplementation of cultured murine epidermal cells with xanthine-xanthine oxidase results in an elevation of ornithine decarboxylase activity, a marker of the second stage of promotion in murine skin (19).

Although there is a strong precedent for a role of reactive oxygen species in tumor promotion, and the *in vivo* promoting activities of TPA analogues correlated with their abilities to elevate epidermal XO activity, any discussion of a role for elevated XO activity in tumor promotion is at this time speculative. The findings that the first stage promoter 4-*O*-methyl-TPA had no effect on XO activity in SENCAR mice, whereas the second-stage promoter mezerein was a very effective XO inducer, suggest that elevation of XO activity is associated with the second stage of promotion as defined with SENCAR mice. Particularly noteworthy are the kinetics of epidermal XO elevation and decline following TPA treatment. The kinetics describing XO activity parallel the kinetics of epidermal hyperplasia and keratinocyte differentiation following a single topical application of TPA (31). Our future studies are directed at determining whether elevated XO activity is associated with promoter-induced epidermal hyperplasia and/or keratinocyte differentiation.

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