ABSTRACT

Topical application of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) to SENCAR mouse skin results within 48 h in a 3-fold elevation of xanthine oxidase (XO) activity, an enzyme capable of generating the reactive oxygen species superoxide and hydrogen peroxide. The antiinflammatory steroid fluocinolone acetonide, an inhibitor of TPA-induced hyperplasia, as well as the multiple stages of tumor promotion as defined in SENCAR mice (Stages I and II), inhibited the TPA-dependent elevation of epidermal XO activity. Neither tosylphenylalanilanyl chloromethyl ketone nor retinoic acid, inhibitors of promotion Stages I and II, respectively, had significant effects on TPA-induced hyperplasia or elevated XO activity. The nonpromoting but hyperplasogenic agents ethyl phenylpropiolate and acetic acid significantly elevated XO activity within 48 h of topical application. The non-phorbol ester tumor promoter benzoyl peroxide also elevated XO activity consistent with the degree of induced hyperplasia. Multiple treatments with TPA or ethyl phenylpropionate resulted in a sustained elevation of XO activity which peaked at five treatments and then declined. Sustained inhibition of XO activity by p.o. administration of allopurinol did not inhibit the TPA-induced hyperplasia as determined histologically. These results suggest that the TPA-dependent elevation of epidermal XO activity is associated with the hyperplasia induced by the agent, and is a consequence of the hyperplasia rather than the cause of it.

INTRODUCTION

XO and XD are purine catabolism enzymes responsible for the conversion of hypoxanthine to xanthine, and xanthine to uric acid. Xanthine dehydrogenase can be converted in vitro to XO by a variety of treatments including limited proteolysis, heating, and treatment with sulfhydryl-modifying reagents (1, 2). This conversion has also been demonstrated to occur in vivo (3, 4). Interferons (3) and ethanol (4) have been reported to elevate murine liver and rat heart XO activity, respectively, by affecting the conversion of XD to XO. As an oxidase XO can use molecular oxygen as an oxidant resulting in production of the ROS superoxide and hydrogen peroxide. The cytotoxicity of interferons and ethanol to the aforementioned tissues has been postulated to be mediated by elevated XO activities and the production of ROS (3, 4).

A substantial data base circumstantially implicates free radicals and ROS in tumor promotion (5-10). Recent work in our laboratory has shown that topical application of a promoting dose of TPA to SENCAR mouse skin elevates epidermal XO activity (11). This elevation was due to a TPA-dependent induction of XD synthesis and conversion of XD to XO. We also demonstrated that XO activity could be correlated with the in vivo promoting capabilities of TPA analogues. In the present study we investigated the relevance of TPA-dependent increases in XO activity to murine skin tumor promotion. As an approach we took advantage of the operational subdivision of promotion into two phases (Stages I and II) in SENCAR mice. The antiinflammatory steroid FA has been shown to inhibit both stages of promotion and TPA-induced hyperplasia (12). The protease inhibitor TPCK and RA have been reported to inhibit Stages I and II of promotion, respectively, without having significant effects on TPA-induced hyperplasia (12). In the current study we examined FA, TPCK, and RA for their abilities to inhibit TPA-dependent elevation of epidermal XO activity. We also surveyed the abilities of the nonphorbol ester tumor promoter benzoyl peroxide, and the nonpromoting but hyperplasogenic agents acetic acid and EPP for their abilities to elevate epidermal XO activity. Additionally, to rule out the possibility that TPA-elevated XO resulted in cytotoxicity, and therefore elicited a regenerative hyperplasia, AP was used to inhibit XO activity and its effect on hyperplasia was assessed.

MATERIALS AND METHODS

Chemicals. Hypoxanthine, xanthine, uric acid, TPCK, RA, FA, and AP were purchased from the Sigma Chemical Company. Radiolabeled [8-14C]hypoxanthine (50-53 mCi/mmol) was purchased from American. Cellulose thin-layer chromatography plates were obtained from the Eastman Kodak Company. TPA was purchased from Chemicals for Cancer Research Inc., Eden Prairie, MN; EPP was purchased from the Aldrich Chemical Company, and benzoyl peroxide was obtained from the J. T. Baker Chemical Company.

Animal Treatment Protocols. Female SENCAR mice (6-7 weeks old, National Cancer Institute, Frederickburg, MD) were used for all experiments. Their backs were shaved 2-5 days before treatment with chemicals. Animals in hair regrowth were not used. Benzoyl peroxide and acetic acid were chosen from those used in previous studies (8, 13). For the inhibitor studies animals were treated topically with either TPA (2 μg), TPA (2 μg) plus TPCK (10 μg), TPA (2 μg) plus RA (10 μg), or TPA (2 μg) plus FA (1 μg) in a total volume of 0.2 ml acetone. Inhibitor doses were determined in a previous study (12). Allopurinol was suspended in H2O and given p.o. (50 mg/kg) in 0.2 ml H2O 1 h before, and 6 and 24 h after TPA or acetone treatments.

To assess multiple treatment effects, 0.2 ml acetone, 2 μg TPA or 14.0 mg EPP in 0.2 ml acetone were applied once, 3, 5, 7, and 9 times (2× per week). Mice were sacrificed for enzyme assays 48 h after the last treatment.

Tissue Preparation. Mice were killed by cervical dislocation at selected intervals. The dorsal skin was excised and the epidermis scraped directly as described previously (11). Epithelial scrapings were homogenized in 0.5 ml of 50 mM NaKPO4, pH 7.0, 0.1 mM EDTA and centrifuged for 15 min at 13,500 x g, and the supernatant fluid recentrifuged for 5 min at 13,500 x g. The resulting supernatant fluid was kept on ice and used in XD/XO assays on the same day without freezing.

Xanthine Dehydrogenase/Oxidase Assay. Xanthine dehydrogenase/oxidase were assayed by a modification of the procedure of Mousson et al. (14). A detailed description of the modified assay has been published (11). Total dehydrogenase plus oxidase (XD + XO) activity
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Table 1 Inhibition of TPA-dependent elevation of xanthine dehydrogenase/oxidase by antipromoting agents

Files were applied topically in 0.2 ml acetone to the shaved dorsal skins of SENCAR mice at the following doses: TPA (2 μg), RA (10 μg), FA (1 μg), and TPCK (10 μg).

<table>
<thead>
<tr>
<th>Agent</th>
<th>Time after treatment (h)</th>
<th>nmol/min/mg</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>48</td>
<td>2.24 ± 0.10*</td>
<td>100</td>
</tr>
<tr>
<td>TPA</td>
<td>48</td>
<td>5.87 ± 0.20*</td>
<td>191</td>
</tr>
<tr>
<td>FA</td>
<td>48</td>
<td>1.95 ± 0.29</td>
<td>87</td>
</tr>
<tr>
<td>FA + TPA</td>
<td>48</td>
<td>2.85 ± 0.33!</td>
<td>107</td>
</tr>
<tr>
<td>TPCK</td>
<td>48</td>
<td>2.61 ± 0.07</td>
<td>117</td>
</tr>
<tr>
<td>TPCK + TPA</td>
<td>48</td>
<td>6.06 ± 0.54</td>
<td>271</td>
</tr>
<tr>
<td>RA</td>
<td>48</td>
<td>2.93 ± 0.30*</td>
<td>131</td>
</tr>
<tr>
<td>RA + TPA</td>
<td>48</td>
<td>8.87 ± 1.21!</td>
<td>233</td>
</tr>
</tbody>
</table>

* Results are expressed as the mean values ± SE of two different experiments employing three animals per treatment.

Significantly different from acetone-treated animals, P < 0.001.

Significantly different from acetone-treated animals, P < 0.05.

4 B. Pence, unpublished results.

Effects of Promotion Inhibitors on XO and XD Activities. Epidermal XO and XD activities were significantly elevated within 48 h of topical TPA treatment (Table 1). We have previously demonstrated that the increased activities were due to enhanced XD synthesis, and conversion of XD to XO (11). Fluocinolone acetonide, a potent inhibitor of promotion Stages I and II, inhibited the TPA-dependent increases in both XO and XD activities (Table 1). The agents TPCK and TPCK + TPA, inhibitors of promotion Stages I and II, inhibited the TPA-dependent increases in both XO and XD activities (Table 1).

Results are expressed as the mean values ± SE of two different experiments employing three animals per treatment.

Table 2 Elevation of xanthine dehydrogenase/oxidase by TPA, nonpromoting hyperplastic agents and a nonphorbol tumor promoter

Agents were applied topically in 0.2 ml acetone to the shaved dorsal skins of SENCAR mice.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (mg)</th>
<th>Time after treatment (h)</th>
<th>nmol/min/mg</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>0.002</td>
<td>48</td>
<td>1.72 ± 0.15*</td>
<td>100</td>
</tr>
<tr>
<td>TPA</td>
<td>1.0</td>
<td>48</td>
<td>5.26 ± 0.33*</td>
<td>306</td>
</tr>
<tr>
<td>EPP</td>
<td>2.5</td>
<td>48</td>
<td>3.29 ± 0.24</td>
<td>282</td>
</tr>
<tr>
<td>Acetone + AP</td>
<td>2.5</td>
<td>48</td>
<td>3.29 ± 0.24</td>
<td>282</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>1.0</td>
<td>24</td>
<td>4.38 ± 0.30*</td>
<td>233</td>
</tr>
</tbody>
</table>

Specific activity 48 h after TPA treatment.

Interfollicular epidermal thickness from basal through granular layer (excluding horny layer) was measured 48 h after TPA treatment. Values, mean ± SE from six animals (10 random measurements per animal).

Results are expressed as the mean values ± SE of two different experiments employing three animals per treatment.

Significantly different from acetone-treated controls, P < 0.001.

Significantly different from acetone-treated controls, P < 0.05.

Significantly different from acetone-treated controls, P < 0.001.

Significantly different from acetone-treated controls, P < 0.05.

Morphological Studies. For assessment of epidermal hyperplasia and edema, strips of dorsal skins from treated animals were preserved in 10% buffered formalin, processed by routine histological procedure, and stained with hematoxylin and eosin. Epithelial thickness was determined by light microscopy employing a reference calibration standard.

Results are expressed as the mean values ± SE of two different experiments employing three animals per treatment.

Significantly different from acetone-treated controls, P < 0.01.

Significantly different from acetone-treated controls, P < 0.05.

Significantly different from acetone-treated controls, P < 0.001.

Significantly different from acetone-treated controls, P < 0.01.

Table 3 Effects of allopurinol treatment on hyperplasia induced by TPA in murine epidermis

Allopurinol (AP) was given p.o. (50 mg/kg) 1 h before and 6 and 24 h following TPA (2 μg) treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of Control</th>
<th>Epidermal thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>100</td>
<td>18.00 ± 0.22</td>
</tr>
<tr>
<td>Acetone + AP</td>
<td>100</td>
<td>18.63 ± 0.24</td>
</tr>
<tr>
<td>TPA</td>
<td>258</td>
<td>41.56 ± 1.12</td>
</tr>
<tr>
<td>TPA + AP</td>
<td>36</td>
<td>42.60 ± 1.46</td>
</tr>
</tbody>
</table>

Specific activity 48 h after TPA treatment.

Quantitatively similar to that produced within 48 h by 0.5 μg TPA. Similar to the findings of a previous report (17), no edema was seen in benzoic peroxide treated epidermis.

Allopurinol Inhibition of XO and XD Activities. It has been suggested that TPA induces a regenerative hyperplasia as a consequence of promoter induced cytotoxicity (18). Reactive oxygen species have been reported to be cytotoxic (5-7), and elevated XO activity has been associated with several pathological conditions (3, 4) in which ROS produced by XO have been postulated to be causative factors in the pathology. Therefore, in order to examine the role of elevated XO activity in TPA-dependent hyperplasia, we surveyed the effects of XD/XO inhibition by AP on promoter-induced hyperplasia. Oral administration of AP significantly decreased basal XO activity, and completely suppressed TPA-dependent elevation of XO activity (Table 3). However, AP treatment had no effect on TPA-induced cell proliferation as revealed by histological comparisons (Fig. 1), and measurement of epidermal thickness (Table 3).

Multiple Treatment Effects on XO and XD Activities. Relative to the level of XO activity induced by a single topical application of TPA, chronic treatment of murine epidermis markedly enhanced XO activity (Fig. 2A). Maximal activity was achieved 48 h after five TPA treatments. Thereafter, epidermal XO activity decreased but remained higher than XO activities in mice chronically treated with acetone. The TPA-dependent increases...
TPA-DEPENDENT ELEVATION OF XANTHINE OXIDASE

Fig. 1. Effects of allopurinol on TPA-induced epidermal hyperplasia. Female SENCAR mice were given allopurinol p.o. (50 mg/kg) 1 h before, and 6 and 24 h after topical treatment with acetone or 2 μg TPA. Mice were sacrificed for tissue preparation and fixation 48 h after topical treatment. Treatment panels are: A, acetone; B, acetone plus allopurinol; C, TPA; D, TPA plus allopurinol. Original magnification was 400 x.

Fig. 2. Kinetics of TPA- and EPP-dependent elevation of XO (A) and XO + XD (B) activities in murine epidermis. Mice were topically treated on Mondays and Thursdays with either acetone (●), or 2 μg TPA (△), or 14 mg EPP (■), and sacrificed 48 h after treatment. Points, mean ± SE of two experiments each containing three animals per treatment.

in XO activity were paralleled by increases in total XD + XO activity (Fig. 2B).

Repetitive treatment of murine epidermis with EPP also elevated XO, and XO + XD activities to levels greater than that obtained by a single treatment (Fig. 2). Like TPA, maximal activity was obtained with five EPP treatments. Thereafter, epidermal XO/XD activities decreased to levels obtained with a single application of EPP.

DISCUSSION

In our previous study (11) we demonstrated that the kinetics of XO elevation and decline following TPA treatment generally parallel the kinetics of epidermal hyperplasia following a single topical application of TPA (18). Several results from our current studies suggest that the elevation in XO activity measured in murine epidermis following TPA treatment is associated with the hyperplasia induced by the promoter. First, agents capable of inducing epidermal hyperplasia also elevated XO activity. Furthermore, the degree of XO elevation could be correlated with the relative degree of hyperplasia induced by the agent. Acetic acid and benzoyl peroxide both produce a
weak hyperplasia relative to EPP and TPA (16, 17) and elevated XO activity less than the more hyperplastic agents. In addition, we previously reported (11) that the in vivo promoting activities of a series of phorbol analogues (TPA > phorbol-12,13-dibenzozate > 4-O-methyl-TPA ≥ phorbol) correlated with their abilities to elevate epidermal XO activities. Coincidently, the relative promoting activities of these agents also correlate with their abilities to induce hyperplasia (19). Second, RA and TPCK, which have little effect on TPA-induced hyperplasia (12), had no inhibitory effects on the TPA-dependent elevation of XO activity. Retinoic acid actually appeared to potentiate TPA-dependent elevation of XO activity. Conversely, FA, which is a potent inhibitor of TPA-induced inflammation and hyperplasia (20), also inhibited TPA-dependent effects on XO activity. Since TPCK is a modest inhibitor of TPA-dependent inflammation, it is unlikely that elevated epidermal XO activity is a consequence of TPA-induced inflammation (21).

An association between elevated epidermal XO activity and epidermal hyperplasia is further substantiated by clinical reports on psoriasis patients (22–24). Psoriasis, a chronic skin condition with some characteristics analogous to the hyperplastic response elicited by TPA, is characterized by hyperuricemia in 30 to 40% of patients with active disease (22). In one clinical study the activity of XO in the psoriatic epidermis of some patients was increased about 5-fold relative to normal epidermis (24). These clinical reports strengthen our contention that elevated XO activity is associated with the epidermal hyperplastic state induced by TPA treatment. However, it should be emphasized that the hyperplastic response involves both increased cellular proliferation, and the subsequent differentiation of a majority of the newly synthesized cells. Consequently, a central question is whether the TPA-dependent elevation in XO activity is associated with the proliferation or differentiation phases of epidermal hyperplasia. The inability of allopurinol to inhibit TPA-dependent induction of epidermal hyperplasia, under conditions in which XO and XD activities are suppressed to levels less than the basal activities, demonstrate that elevated XO activity is not necessary for TPA-dependent induction or maintenance of hyperplasia. This conclusion is also supported by the kinetics of XO induction following TPA treatment. Significant elevations in XO activity were not detected until 24 h after TPA treatment, and maximal activity was not reached until 48–96 h after TPA treatment. In contrast, epidermal DNA synthesis occurs within 18 h of TPA treatment (25). Consequently, DNA synthesis precedes significant elevations in XO activity. Collectively, our findings suggest that the induction of XO by TPA is a consequence of hyperplasia, and is not associated with the processes involved in the initiation and maintenance of cellular proliferation. Our current studies are directed at evaluating XO activity as a marker for keratinocyte maturation and differentiation. Preliminary studies on epidermal cells separated by Percoll density centrifugation into populations differing in their stages of differentiation suggest that the XD to XO conversion occurs during the terminal differentiation of keratinocytes.

Reactive oxygen species have been circumstantially implicated in the mechanism of TPA-dependent promotion in murine skin (5–10). The sources of ROS in murine epidermis are unknown. Studies using allopurinol to inhibit XO activity have implicated XO-derived ROS as a causative factor in several pathological conditions (3, 4). It is conceivable that XO may be a source of epidermal ROS. However, the significance of elevated XO activity to murine skin tumor promotion is speculative, especially in light of the effects of EPP on epidermal XO activity. Repetitive treatments with EPP resulted in a sustained elevation of XO activity that was kinetically and quantitatively similar to that obtained with chronic TPA treatment (Fig. 2). Because EPP is considered to be a nontumor-promoting agent (26), this finding suggests that the induction of XO by TPA is not relevant to the processes of promotion. However, it should be emphasized that the effects of ROS are ultimately concentration dependent, and ROS concentration is modulated by a balance between those systems responsible for their production and detoxification. Several investigators have reported that a topical application of TPA decreases the specific activities of the ROS detoxification enzymes SOD and catalase (27–29). Furthermore, a sustained repression of epidermal SOD activity is maintained by repetitive TPA treatment (29). In contrast, EPP has been reported to have no effects on epidermal SOD and catalase activities (27). Consequently, relative to EPP-treated epidermis, a higher concentration of ROS might accumulate in TPA epidermis owing to an increase in ROS generation and a decrease in the activities of ROS detoxification enzymes. The contribution of XO to TPA-dependent promotion is currently under investigation.

REFERENCES

TPA-DEPENDENT ELEVATION OF XANTHINE OXIDASE


Murine Epidermal Xanthine Oxidase Activity: Correlation with Degree of Hyperplasia Induced by Tumor Promoters

Barbara C. Pence and John J. Reiners, Jr.


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