Induction of the Polyamine-biosynthetic Enzymes in Mouse Epidermis by Tumor-promoting Agents

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SUMMARY

A single topical application of 1.0 mg of croton oil or 17 nmoles of 12-0-tetradecanoyl-phorbol-13-acetate (TPA) resulted in a rapid, transient stimulation of mouse epidermal ornithine decarboxylase activity. The activity reached a peak (230-fold greater than control after TPA) at 4 to 5 hr after croton oil or TPA treatment and returned to control level by 12 hr. The stimulation of S-adenosyl-L-methionine decarboxylase activity was less pronounced, reaching a peak of activity (6- to 7-fold greater than control) at 9 to 12 hr after TPA or croton oil and slowly declining to control level. The stimulation of both enzyme activities was dependent on the dose of TPA applied and correlated well with the promoting ability of these doses on mouse skin. Phorbol, the nonpromoting parent alcohol of TPA, did not affect the enzyme activities.

Cycloheximide pretreatment abolished the increase in enzyme activities after TPA application. By measuring the decline of enzyme activity following cycloheximide treatment, enzyme half-lives of 17 and 41 min were obtained for ornithine and S-adenosyl-L-methionine decarboxylase, respectively. 5-Azacytidine pretreatment prevented the stimulation of enzyme activities by TPA, while actinomycin D had no effect. Cordycepin (3'-deoxyadenosine) partially blocked the rise in enzyme activities.

INTRODUCTION

In recent years, a large body of evidence has accumulated concerning the important role of L-ornithine decarboxylase (EC 4.1.1.17) and S-Ado-Met decarboxylase (EC 4.1.1.50) in eukaryotic tissues undergoing rapid proliferation in response to various stimuli. The activities of these enzymes and the levels of their biosynthetic products, putrescine and spermidine, are greatly increased in regenerating rodent livers following partial hepatectomy (22, 27, 28), in lymphocytes after mitogen stimulation (14), in the mouse salivary gland following isoproterenol administration (12), and in various target organs after appropriate hormonal treatments (6, 20, 34). In addition, ornithine decarboxylase activity is elevated in fast-growing neoplasms such as Morris hepatomas 3924A and 7777 (33) and L1210 leukemic cells (26).

The experimental induction of mouse skin tumors can be separated into at least 2 stages, initiation and promotion (19). Following initiation with a single subcarcinogenic dose of a chemical such as 7,12-dimethylbenz[a]anthracene, tumors can be elicited with repetitive topical applications of croton oil or certain phorbol esters (1, 3). The sequence of biochemical events in mouse epidermis after a single application of croton oil or a promoting phorbol ester is very similar to those systems (described above) undergoing rapid proliferation following appropriate stimuli. Thus, the incorporation of 32P into skin phospholipids is stimulated 2 to 3 hr after promoter treatment (24), followed by a sequential stimulation of RNA, protein, and DNA synthesis (2). In addition, tumor promoters stimulate the phosphorylation of epidermal histones (23). These events appear to be characteristic of cell populations stimulated to proliferate and have led to the hypothesis that the mechanism of promotion involves gene activation (4). Since greatly increased levels of ornithine and S-Ado-Met decarboxylase activities are a prominent feature of rapidly proliferating tissues (25), we decided to measure the activities of these enzymes in mouse epidermis following treatment with croton oil and TPA, the most active promoting agent found in croton oil (10). We wanted to answer the following questions: (a) are specific genes activated following the application of a tumor promoter? and (b) what is the mechanism of this activation?

The discovery of what specific genes are activated (or turned off) after promoter treatment, and how this is accomplished, will hopefully lead to an understanding of which biochemical responses of the epidermis are essential for promotion.

MATERIALS AND METHODS

Animals. Female Charles River CD-1 mice were obtained from Charles River Breeding Laboratories, Wilmington, Mass., and were used when 7 to 9 weeks of age. The dorsal hair of each mouse was shaved with surgical clippers at least 1 to 2 days before use, and only those mice showing no hair regrowth were used. All animals received water and Wayne Breeder Blox (Allied Mills, Chicago, Ill.) ad libitum throughout the experimental period. Mice were routinely killed between 1 and 3 p.m. to avoid variations due to circadian rhythms.

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1 This work was supported in part by Grant BC-14 from the American Cancer Society and Grants CA-07175 and CA-05002 from the National Cancer Institute.

2 The abbreviations used are: S-Ado-Met, S-adenosyl-L-methionine; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

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Chemicals. All chemicals applied to mouse skin were dissolved in reagent grade acetone and delivered in a volume of 0.15 or 0.20 ml. Chemicals that were injected i.p. were dissolved immediately before use in 0.9% NaCl solution and administered in a volume of 0.2 ml within 10 min.

Croton oil was obtained from Amend Drug and Chemical Co., Irvington, N. J. TPA was purchased from Consolidated Midland Corp., Brewster, N. Y. Phorbol was prepared in this laboratory by Dr. W. M. Baird. Actinomycin D, 5-azacytidine, cordycepin, and cycloheximide were obtained from Sigma Chemical Co., St. Louis, Mo. Putrescine dihydrochloride was from Calbiochem, San Diego, Calif.

DL-[1-14C]Ornithine (58 mCi/mmmole) was obtained from Amersham/Searle Corp., Arlington Heights, Ill. DL-[2-14C]Ornithine (3.87 mCi/mmmole) was from ICN Corp., Irvine, Calif., and S-adenosyl-L-[carboxyl-14C]methionine (7.7 mCi/mmmole) and L-[4,5-3H]leucine (44.2 Ci/mmmole) were obtained from New England Nuclear, Boston, Mass.

Preparation of Epidermal Extracts. In order to obtain sufficient material, the dorsal skins from each of 4 to 5 mice were pooled to constitute each treatment group. The epidermis was obtained by the method of Raineri et al. (23) with minor modifications. Briefly, the mice were killed by cervical fracture, and the depilatory agent, Nudit (Helena Rubinstein, New York, N. Y.), was applied to the shaved area of the back. After 5 min, the Nudit was washed off with cold tap water, and the skin was excised and plunged into ice-cold water. The skin was then placed in 55°C water for 30 sec. The skin was placed epidermis side up on a cold glass plate, and the epidermal scrapings (treated 4 hr earlier with 17 nmoles TPA) were vigorously scraped with a razor blade. This procedure yields an excellent separation of epidermis from dermis, as verified by light microscopy.

The pooled epidermal sheets were homogenized in 50 mM sodium phosphate buffer, pH 7.2, containing 0.1 mg pyridoxal phosphate and 0.1 mM EDTA, in a Polytron PT-10 homogenizer for 15 to 20 sec at 0–4°C. The supernatant fraction obtained after centrifugation at 30,000 × g for 30 min at 0°C was used for estimation of enzyme activities.

The effects of Nudit and the 55°C heat treatment on enzyme activities were assessed in the following manner. Instead of preparing epidermis in the usual way, skins (treated 4 hr earlier with 17 nmoles TPA) were vigorously scraped with a razor blade, and the epidermal scrapings were homogenized and centrifuged as usual. The supernatant obtained was assayed without further treatment or after heating at 55°C for 25 sec. Both ornithine and S-Ado-Met decarboxylase activities were assessed in the following manner. After 5 min, the Nudit was washed off with cold tap water, and the skin was excised and plunged into ice-cold water. The skin was then placed in 55°C water for 30 sec. The skin was placed epidermis side up on a cold plate and the epidermis was scraped off with a razor blade. This procedure yields an excellent separation of epidermis from dermis, as verified by light microscopy.

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Ornithine Decarboxylase Assay. Enzyme activity was determined by measuring the release of 14CO2 from DL-[1-14C]Ornithine, essentially as described by Russell and Snyder (28). Incubations were carried out in 25-ml Erlenmeyer flasks equipped with a rubber stopper-polyethylene center well assembly (Kontes Glass Co., Vineland, N. J.) containing 0.2 ml ethanolamine:2-methoxyethanol (2:1, v/v). Incubation mixtures consisted of 0.4 μmole pyridoxal phosphate, 1.0 μmole diithiothreitol, 0.2 μmole L-ornithine, 100 μmoles sodium phosphate, pH 7.2, 0.1 to 0.6 ml epidermal extract, and approximately 0.5 μCi DL-[1-14C]Ornithine (58 mCi/mmmole) in a final volume of 2.0 ml. All components except DL-[1-14C]Ornithine were incubated at 37°C for 10 min prior to the addition of isotope. Incubations were routinely carried out for 30 min at 37°C. The reaction was stopped by addition of 1.0 ml 2 M citric acid and incubations were continued for an additional 30 min to insure complete absorption of 14CO2. The center wells were removed and cut directly into scintillation vials containing 2 ml ethanol and 10 ml of a toluene-based scintillant consisting of 4 g PPO and 50 mg POPOP per liter of toluene. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter at 80% efficiency. All enzyme activities were corrected against a blank prepared by heating epidermal extracts at 100°C for 10 min and assaying as above. Activity was expressed as nmoles CO2 in 30 min per mg protein.

Enzyme activity was proportional to the amount of extract added in the range 0.1 to 2.0 mg protein and to time of incubation up to 60 min. The substrate concentration routinely used (100 μM) was nonsaturating, but in some experiments a saturating substrate concentration (2 mM) was used and similar results were obtained.

Determination of the Stoichiometry of Putrescine Formation and CO2 Release from Ornithine. The standard ornithine decarboxylase assay conditions were used with these exceptions: (a) 12-ml screw-cap centrifuge tubes were used instead of Erlenmeyer flasks; (b) DL-[2-14C]Ornithine was substituted for DL-[1-14C]Ornithine in the incubation mixtures; and (c) 0.5 ml 10% HCl10 was used to stop the reaction.

Following addition of HC104, the tubes were kept at 0°C for 15 min and then centrifuged at 2000 × g for 10 min at 0°C. The supernatants were poured off and the pellets were reextracted twice with 2.5 ml 2% HCl10, followed by centrifugation. The supernatants were combined and applied to 6-cm Dowex 50 columns, and the polyamines were eluted as described by Inoue and Mizutani (11). The elution procedure results in a polyamine-containing fraction free of basic amino acids, with 97% recovery of the polyamines. The fractions containing the polyamines (in 6 N HCl) were evaporated to dryness on a rotary evaporator at 60°C, the residues were dissolved in 0.1 N HCl, and radioactivity was determined by liquid scintillation counting in 10 ml Scintisol (Isolab Inc., Akron, Ohio).

S-Ado-Met Decarboxylase Assay. Enzyme activity was determined by measuring the release of 14CO2 from S-adenosyl-L-[carboxyl-14C]methionine in the presence of added putrescine as an acceptor of the propylamino moiety derived from decarboxylated S-Ado-Met (9). Incubations were carried out, and the radioactivity was measured as described above for the ornithine decarboxylase assay, except 15-ml centrifuge tubes were used instead of Erlenmeyer flasks, and 0.5 ml of 2 M citric acid was used to stop the reaction. The incubation medium consisted of 0.01 μmole pyridoxal phosphate; 0.5 μmole putrescine; 0.020 μmole S-adenosyl-L-[carboxyl-14C]methionine (approxi-

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mately 0.2 μCi/tube); 10 μmoles sodium phosphate, pH 7.2; and 0.05 to 0.10 ml epidermal extract in a final volume of 0.20 ml. The decarboxylation of S-Ado-Met by epidermal extracts was negligible in the absence of putrescine.

The concentration of S-Ado-Met used (100 μM) was nonsaturating, but in experiments in which a saturating substrate concentration (2 mM) was used, similar changes in enzyme activities were observed. The enzyme activity was proportional to the amount of epidermal extract added in the range 0.05 to 0.30 mg protein and to the time of incubation up to 60 min.

**Protein Determination.** The protein concentration of the epidermal extracts was measured by the method of Lowry *et al.* (18), with bovine serum albumin as standard.

**Determination of the Incorporation of 3H-labeled Leucine into Total Epidermal Protein following Cycloheximide Treatment.** Groups of 4 mice were given i.p. injections of 0.9% NaCl solution or cycloheximide (70 mg/kg) dissolved in 0.9% NaCl solution. At various times after treatment, 100 μCi 3H-labeled leucine were injected and the mice were killed 70 min later. The mice were quickly weighed, and the skins were excised, spread dermis side down on a piece of filing card, and placed in a beaker of 1% acetic acid overnight at 4°C. After removing the skins from acetic acid, the epidermis was scraped off with a razor blade. Protein was then isolated from the pooled epidermal sheets exactly as described by Baird *et al.* (2). The specific activity of each treatment group was expressed as dpm per μg protein per μCi injected per g body weight.

**RESULTS**

**Stoichiometry of the Ornithine Decarboxylase Assay.** In order to determine that the decarboxylation of L-ornithine leads to the stoichiometric production of CO2 and putrescine, the amounts of 14CO2 and 14C-labeled putrescine derived from DL-[1-14C]ornithine and DL-[2-14C]ornithine, respectively, were measured. Identical aliquots of an epidermal extract from mice treated for 5 hr with 17 nmoles TPA produced 1.425 μmoles of CO2, while 1.433 μmoles of putrescine were obtained. Thus, interfering reactions utilizing labeled ornithine leading to the formation of 14CO2 were not present in detectable amounts in the epidermal extracts.

**Effect of Croton Oil, TPA, and Phorbol.** A single application of 1.0 mg of croton oil to mouse skin resulted in a rapid and transient stimulation of ornithine decarboxylase activity. As shown in Chart 1, the peak of activity (approximately 100-fold greater than the acetone-treated control) occurred 4.5 hr after application and rapidly declined to control level by 9 hr where it remained for up to 24 hr. Enzyme activity was also measured at 2, 4, and 7 days after treatment and no increase over the control level was observed, although a transient rise in activity similar to that occurring at early times after treatment could easily have been missed. The response of S-Ado-Met decarboxylase activity was less pronounced; the activity rose slowly to a peak (6-fold greater than the acetone-treated control) around 9 hr after application and slowly declined. The activity was 2- to 3-fold greater than control at 4 days and had returned to the control level by 7 days.

Since croton oil is a complex mixture containing triglycerides, other lipids, and various promoting and hyperplastic agents for mouse skin, the effect of croton oil on the activities of the polyamine biosynthetic enzymes is a summation of the effects of all compounds present in croton oil. Therefore, we studied the effect of a pure tumor promoter, TPA, the most active tumor promoter found in croton oil (10), on these enzyme activities. The response of epidermal ornithine and S-Ado-Met decarboxylase activities to a single topical application of 17 nmoles TPA is shown in Chart 2. The ornithine decarboxylase activity rapidly increased, reaching a peak 5 hr after application (230-fold greater than control) and quickly returned to control levels by 12 hr where it remained for up to 24 hr.

**Chart 1.** The effect of a single topical application of 1.0 mg croton oil on the activities of epidermal ornithine decarboxylase and S-Ado-Met decarboxylase. Groups of 5 mice were treated with either 0.2 ml acetone or croton oil dissolved in acetone and were killed at the times indicated. Points, means of triplicate determinations of enzyme activity (variation < 10%). The activity of ornithine decarboxylase in control epidermis was extremely low and variable, so 1 typical experiment is presented. prot., protein.

**Chart 2.** The effect of a single topical application of 17 nmoles TPA on the activities of epidermal ornithine decarboxylase and S-Ado-Met decarboxylase. Groups of 5 mice were treated with either 0.2 ml acetone or TPA dissolved in acetone and killed at the times indicated. Points, means of triplicate determinations of enzyme activity (variation < 10%). The activity of ornithine decarboxylase in control epidermis was extremely low and variable, so 1 typical experiment is presented. prot., protein.
Enzyme activity was also measured at 2, 4, and 7 days after treatment and no increase over the control level was seen, although a transient rise in activity similar to that occurring at early times after treatment could easily have been missed. The S-Ado-Met decarboxylase activity also increased rapidly soon after TPA application, showing a broad peak of activity (5- to 7-fold greater than control) at 9 to 15 hr and slowly declining to near control levels by 4 days. Thus, the response of S-Ado-Met decarboxylase activity to croton oil differed from that to TPA in 2 respects: the peak of activity following TPA treatment was much broader, yet the activity had returned to approximately the control level by 4 days, at which time the croton oil-treated epidermis showed an enzyme activity 2- to 3-fold greater than control. This differential response to croton oil and TPA may be due to uncertainty in the precise amount of TPA present in the batch of croton oil used or the effect of other compounds present in croton oil.

We also tested the effect of phorbol, the alcohol from which TPA is derived and which is completely inactive as a skin tumor promoter (2), on these 2 enzyme activities. A single application of a dose equimolar to the TPA dose used produced no change in ornithine or S-Ado-Met decarboxylase activities at any time studied, up to 48 hr after application (the longest time point tested).

The increases in enzyme activities following TPA or croton oil application were not due to the presence in the epidermal extracts of inhibitors or activators of the enzyme activities, since mixing extracts from control and promoter-treated mice always gave additive specific activities.

**Dose Dependency.** The effect of various doses of TPA on the stimulation of enzyme activities is shown in Chart 3. The doses, 17, 1.7, and 0.17 nmoles, have been described as very good, good, and weak promoting doses, respectively, of this compound for mouse skin (1). In a 2-stage tumorigenesis experiment, the yield of papillomas produced by repetitive applications of these doses was 11 papillomas/mouse for the highest dose, 2.3 papillomas/mouse for the intermediate dose, and only 0.3 papilloma/mouse for the lowest dose. The activity of ornithine decarboxylase 4 hr after application of these doses was stimulated roughly 130-fold after 17 nmoles TPA, 60-fold after 1.7 n mole, and 7-fold after 0.17 n mole. The S-Ado-Met decarboxylase activity, measured at 24 hr after application of these doses, was increased in a similar dose-dependent manner. Thus, the magnitude of the stimulation of these enzyme activities is closely correlated with the effectiveness of each dose as a tumor promoter in mouse skin.

**The Role of Protein Synthesis.** In order to investigate the role of protein synthesis in the stimulation of enzyme activities following promoter treatment, we have determined the conditions under which maximal in vivo inhibition of epidermal protein synthesis following cycloheximide treatment occurs. We have found that a single dose of 70 mg/kg, administered i.p., rapidly inhibits the incorporation of 3H-labeled leucine into epidermal protein, as measured by the method of Baird et al. (2). Chart 4 shows that, within 15 min after injection, incorporation of 3H-labeled leucine was reduced to 5% of control. The inhibition slowly faded; at 2 hr after cycloheximide treatment, the inhibition was only 35%.

The effect of cycloheximide on TPA-stimulated ornithine and S-Ado-Met decarboxylase activities is shown in Table 1. Treatment with cycloheximide 30 min prior to TPA application nearly abolished the stimulation of enzyme activities measured 4 hr after 17 nmoles TPA. However, when the interval between TPA treatment and killing was extended to 24 hr with cycloheximide again given 30 min before TPA, no reduction in S-Ado-Met decarboxylase activity was observed. Thus, it appears that, once the block of protein synthesis by cycloheximide has been removed (within a few hr after cycloheximide injection), the increase in S-Ado-Met decarboxylase activity could still be expressed. In contrast, the dramatic increase in ornithine

![Graph showing enzyme activity vs. dose of TPA](image)

**Chart 3.** The dose response of a single topical application of 0.17, 1.7, and 17 nmoles TPA on epidermal ornithine decarboxylase and S-Ado-Met decarboxylase activities. Groups of 4 mice were treated with the indicated dose of TPA and killed 4 hr later (for ornithine decarboxylase determination) or 24 hr later (for S-Ado-Met decarboxylase determination). Points, means of triplicate determinations of enzyme activity (variation < 10%).
decarboxylase activity was not observed in animals given cycloheximide 30 min prior to TPA by extending the interval between TPA treatment and the time of killing. When enzyme activity was measured at 6, 8, 12, 16, 20, and 24 hr after TPA treatment, only at 8 hr was the activity above control values. However, even at this time point, the enzyme level was depressed 40% compared to its 0.9% NaCl solution-injected, TPA-treated control.

In another type of cycloheximide experiment, the in vivo half-lives of these 2 enzymes were estimated by measuring the decline in enzyme activities following cycloheximide administration. The half-life of epidermal ornithine decarboxylase derived by this method was approximately 17 min, as shown in Chart 5B. Chart 5A illustrates that the half-life of epidermal S-Ado-Met decarboxylase is roughly 41 min. These values are quite similar to those reported for other eukaryotic tissues (14, 29, 30), and they represent the shortest known half-lives for any eukaryotic enzyme. When cycloheximide was added directly to incubation mixtures, no inhibition of either enzyme activity was observed, even at a concentration of 1 mM. The half-lives were determined for the TPA-stimulated enzymes; due to the low basal values, the half-lives of these enzymes could not be reliably estimated in normal epidermis.

These results suggest that the increases in the activities of the polyamine biosynthetic enzymes result from increased de novo synthesis of enzyme molecules.

The Role of RNA Synthesis. In order to assess the role of RNA synthesis in the stimulation by TPA of ornithine and S-Ado-Met decarboxylase activities, the effect of actinomycin D was studied. Table 2 shows the results of experiments in which a high dose of actinomycin D (100 µg/mouse) was applied topically 30 min prior to TPA application, and the enzyme activities were measured 4 or 24 hr later. The drug was not effective in preventing the rise in enzyme activities seen at 4 hr after application, while a slight (~21%) reduction of S-Ado-Met decarboxylase activity was observed 24 hr after TPA treatment. In other experiments, we have investigated the effect of dose, route of administration, and the timing of drug treatment relative to TPA application and have failed to demonstrate any substantial inhibition of either enzyme activity by actinomycin D.

To investigate further the role of RNA synthesis in the increased enzyme synthesis, we studied the effect of 5-azacytidine on this phenomenon. This analog of cytidine is incorporated into RNA (13, 31) and DNA (17), depresses the hormonal induction of various enzymes (5, 16), and has shown antileukemic and carcinostatic properties (17, 32). We administered various doses of the analog by i.p. injection 30 min before TPA application and measured ornithine and S-Ado-Met decarboxylase activities 4 and 24 hr later (Table 3). The ornithine decarboxylase activity was reduced to 10% of 0.9% NaCl solution-injected control animals by a dose of 1.5 mg/mouse; lower doses were only slightly inhibitory. The S-Ado-Met decarboxylase activity was much more sensitive to inhibition by the drug; at 4 hr after TPA, enzyme activity was strikingly reduced in a

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**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme specific activity (nmoles CO₂ in 30 min/mg protein)</th>
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<tbody>
<tr>
<td>TPA</td>
<td>Cycloheximide</td>
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<tr>
<td></td>
<td>Ornithine decarboxylase</td>
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<tr>
<td>-</td>
<td>-</td>
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<td>*</td>
<td>-</td>
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</table>

*Mice were treated with TPA 4 hr before killing.*

*Nice were treated with TPA 24 hr before killing.*

*ND, not determined.*

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**Chart 5. Decline of enzyme activities following cycloheximide administration.** Groups of 5 mice were given i.p. injections of either 0.2 ml 0.9% NaCl solution or cycloheximide (70 mg/kg) dissolved in 0.9% NaCl solution and were killed at the times indicated. All mice had been treated with 17 nmoles TPA either 4 hr before killing (for ornithine decarboxylase determination) or 24 hr before killing (for S-Ado-Met decarboxylase determination). Points, means of triplicate determinations of enzyme activity (variation <10%). prot., protein.
dose-dependent manner (Table 3). If the duration of TPA treatment was increased to 24 hr, enzyme activity was still reduced at the highest dose tested.

The results of experiments in which 5-azacytidine was administered after TPA treatment are shown in Table 4. If the drug was given 1 hr before killing to animals treated 3 hr earlier with 17 nmoles TPA, no effect on either ornithine or S-Ado-Met decarboxylase activity was observed. However, if the analog was injected 2 hr before killing, both enzyme activities were reduced dramatically. This effect of 5-azacytidine was observed only at early times after TPA treatment, since drug treatment 2 hr before killing in animals treated 22 hr earlier with TPA only slightly reduced S-Ado-Met decarboxylase activity.

The inhibitory action of 5-azacytidine on the enzyme activities was not due to a direct inhibition of either enzyme, since addition of the drug to incubation mixtures had no effect on either enzyme activity, even at a concentration of 1.0 mM. We have also studied the effect of a high dose of the drug (1.5 mg/mouse) on total protein synthesis, as determined by the incorporation of ^3H-labeled leucine into epidermal protein. 5-Azacytidine only slightly inhibited (25% or less) the incorporation of ^3H-labeled leucine into protein at all times up to 3 hr after drug administration (data not shown). Thus, it is unlikely that the effects of the analog on enzyme activity are due to a general inhibition of protein synthesis in the epidermis.

An indication that increased transcriptional activity may be at least partially responsible for the stimulation of enzyme activities was obtained from experiments with cordycepin (3'-deoxyadenosine). Recent work has demonstrated the presence of polyadenylate sequences up to 200 nucleotides long on heterogeneous rRNA and cytoplasmic mRNA of eukaryotic cells (7, 15). Cordycepin appears to specifically inhibit the polyadenylation reaction, with little or no effect on the synthesis of rRNA and heterogeneous mRNA (21). Table 5 shows the results of a typical experiment in which cordycepin, administered to mice following TPA treatment, reduced the levels of ornithine and S-Ado-Met decarboxylase. The reduction of ornithine decarboxylase activity was slight but reproducible, while the inhibition of S-Ado-Met decarboxylase activity was more evident. The experiment has been repeated twice with similar results. The drug does not inhibit total RNA synthesis in the epidermis at the dose used (T. G. O'Brien and R. K. Boutwell, unpublished data), and addition of the drug directly to

### Table 2

**The effect of actinomycin D on the stimulation by TPA of mouse epidermal ornithine and S-Ado-Met decarboxylase activities**

<table>
<thead>
<tr>
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<td></td>
<td>Ornithine</td>
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<td>TPA 4 hr</td>
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</tr>
<tr>
<td>Actinomycin D</td>
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<td>+</td>
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<td>+</td>
<td>-</td>
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* Mice were treated with TPA 4 hr before killing.
* Mice were treated with TPA 24 hr before killing.
* ND, not determined.

### Table 3

**The effect of 5-azacytidine pretreatment on the stimulation by TPA of mouse epidermal ornithine and S-Ado-Met decarboxylase activities**

<table>
<thead>
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<th>Treatment</th>
<th>Dose of 5-azacytidine (mg)</th>
<th>Enzyme specific activity (nmoles CO₂ in 30 min/mg protein)</th>
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<tr>
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<td>1.50</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mice were treated with TPA 4 hr before killing.
* Mice were treated with TPA 24 hr before killing.
* ND, not determined.

### Table 4

**The effect of 5-azacytidine, administered after TPA treatment, on the levels of ornithine and S-Ado-Met decarboxylase**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme specific activity (nmoles CO₂ in 30 min/mg protein)</th>
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<td>+</td>
<td>(2)</td>
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<tr>
<td>+</td>
<td>(1)</td>
</tr>
<tr>
<td>+</td>
<td>ND</td>
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<tr>
<td>+</td>
<td>ND</td>
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<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, hours between injection of 0.9% NaCl solution or 5-azacytidine (1.5 mg/mouse) dissolved in 0.9% NaCl solution. Epidermal extracts were prepared and enzyme assays were carried out as described in "Materials and Methods." Results are expressed as the mean of triplicate determinations of enzyme activities (variation < 10%).

* Mice were treated with TPA 4 hr before killing.
* Mice were treated with TPA 24 hr before killing.
* ND, not determined.

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incubation mixtures has no effect on either ornithine or S-Ado-Met decarboxylase, even at a concentration of 1.0 mM.

**DISCUSSION**

The sequence of biochemical changes occurring in mouse epidermis following a single application of croton oil or TPA has been shown to include: increased incorporation of \(^{32}P\) into phospholipids (24); increased phosphorylation of histones (23); a sequential stimulation of RNA, protein, and DNA synthesis (2); and finally, cell division. We report here the rapid, transient rise in the polyamine biosynthetic enzymes, ornithine decarboxylase, and S-Ado-Met decarboxylase, after a single application of an active promoting agent. The increase in enzyme activities appears to be due to specific gene activation in the epidermis, which may be the essential function of promoters (4).

The response of epidermal ornithine decarboxylase to a single application of croton oil or TPA is quite striking: from a barely detectable basal level, the activity rises, after a lag period of about 2 hr, to a peak around 5 hr after application and then quickly declines to the basal level by 9 hr. The magnitude and rapidity of this stimulation (230-fold from a barely detectable basal level, the activity rises, after a lag period of about 2 hr, to a peak around 5 hr after application and then quickly declines to the basal level by 9 hr) are so similar (compare Charts 1 and 2), the response to other, nonpromoting compounds present in croton oil. This conclusion is strengthened by the correlation between the response of these enzymes to different doses of TPA and the promoting ability of these doses. Moreover, phorbol, the nonpromoting parent alcohol of TPA, has no effect on either of the 2 enzymes studied. Thus, it appears that enhanced ornithine and S-Ado-Met decarboxylase activity is one of the earliest and largest responses of mouse epidermis to promoter treatment. This response occurs before the 3- to 4-fold increases in total RNA and protein synthesis observed after TPA or croton oil treatment. In fact, ornithine decarboxylase activity had returned to the basal level when RNA and protein synthesis were at a maximum (2).

The stimulation of enzyme activities appears to be due to increased de novo synthesis, as suggested by the results with cycloheximide, an efficient in vivo inhibitor of epidermal protein synthesis. Administration of the drug shortly before promoter application abolished the stimulation of enzyme activities that occurred within 4 hr after treatment; when the enzyme activities were measured at longer times after treatment, ornithine decarboxylase activity never attained the high level seen in control mice, while S-Ado-Met decarboxylase activity at 24 hr after TPA application was similar in cycloheximide-treated and control mice (Table 1). These results suggest that the large stimulation of ornithine decarboxylase activity can only occur within a few hr after promoter treatment, while S-Ado-Met decarboxylase activity, although depressed soon after cycloheximide treatment, increases once the block of protein synthesis is removed.

The short half-lives of these enzymes, approximately 17 min for ornithine decarboxylase and 41 min for S-Ado-Met decarboxylase, indicate an extremely rapid intracellular turnover of these enzymes. The determination of enzyme half-lives by measuring the decline in enzyme activities following cycloheximide treatment, however, assumes that the activity measured is proportional to the actual amount of enzyme protein present and that cycloheximide does not alter the normal rate of enzyme degradation. Until suitable radioimmunological experiments can be done, the values obtained should be considered tentative. In any event, a short half-life for ornithine decarboxylase could account for the rapid decline in enzymatic activity starting about 5 hr after promoter treatment. We cannot eliminate the possibility that the half-lives of these enzymes in promoter-stimulated epidermis are different than the half-lives in normal epidermis; it is clear, however, that changes in the half-lives alone cannot account for the vast increases in enzyme activities.

The involvement of transcriptional events in the stimulation of enzyme activities is uncertain. The failure of actinomycin D to block the response to promoters could be due to the lack of effect of the drug on epidermal mRNA synthesis, as suggested by Flamm et al. (8). In their study, doses of actinomycin D that maximally inhibited RNA synthesis had no effect on epidermal protein synthesis. Since the effects of croton oil and TPA, a pure promoter, are so similar (compare Charts 1 and 2), the response to croton oil is probably related to its promoting ability, rather than being a response to other, nonpromoting compounds present in croton oil. This conclusion is strengthened by the correlation between the response of these enzymes to different doses of TPA and the promoting ability of these

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**Table 5**

The effect of cordycepin on the stimulation by TPA of mouse epidermal ornithine and S-Ado-Met decarboxylase activities

Groups of 4 mice were treated with 17 nmoles TPA and killed 6 hr later. At various times before killing, 0.9% NaCl solution or cordycepin (0.5 mg/mouse) dissolved in 0.9% NaCl solution was injected i.p. Epidermal extracts were prepared and enzyme assays were carried out as described in "Materials and Methods." Results are expressed as the mean of triplicate determinations of enzyme activities (variation <10%).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme specific activity (nmoles CO₂ in 30 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>TPA</td>
<td>Cordycepin*</td>
</tr>
<tr>
<td>− (2)</td>
<td>0.019</td>
</tr>
<tr>
<td>+ (2)</td>
<td>2.726</td>
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<tr>
<td>+ (3)</td>
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<tr>
<td>+ (2)</td>
<td>1.758</td>
</tr>
<tr>
<td>+ (1)</td>
<td>2.392</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, hours between injection of 0.9% NaCl solution or cordycepin and killing.
treatment in vivo indicate that new mRNA synthesis may be at least partially required for optimal enzyme synthesis. In a recent study, Heby and Russell (9) found that 5-azacytidine, a potent antileukemic agent, suppressed the rise in ornithine and S-Ado-Met decarboxylase activity, and the concomitant accumulation of polyamines, in the spleens of mice given inoculations of L1210 leukemic cells. When the drug treatment was stopped, a rapid increase in enzyme activities and polyamine accumulation ensued. We found a dose-dependent inhibition of ornithine and S-Ado-Met decarboxylase synthesis when 5-azacytidine was administered 30 min before promoter treatment. From the kinetics of nucleic acid synthesis in the epidermis following a single application of an active promoter, we feel that the inhibition of enzyme synthesis by the drug could be due to its incorporation into specific mRNAs, resulting in mistranslation or greatly increased lability of the mRNAs coding for ornithine and S-Ado-Met decarboxylases. In the 1st 6 hr after TPA application, the incorporation of 3H-labeled thymidine into epidermal DNA is depressed, while the incorporation of precursors into RNA is stimulated (2). Thus, if the effect of 5-azacytidine on enzyme synthesis is due to its incorporation into nucleic acid, an RNA species is the most likely target. We cannot rule out the possibility that, instead of its incorporation into mRNA, the incorporation of the analog into another RNA species necessary for translation is responsible for its effect on enzyme synthesis, such as the terminal CCA of tRNA. However, such an RNA species must not be essential for the translation of the majority of epidermal proteins, since the drug only slightly inhibits the incorporation of 3H-labeled leucine into total protein.

The inhibition of enzyme synthesis by cordycepin also suggests that the increased transcription of mRNA is at least partially responsible for the increased levels of these enzymes after promoter treatment. Although no precise function has been assigned to the polyadenylate sequences on mRNA, their presence on all eukaryotic mRNAs examined so far, except histone mRNA, suggests that these sequences may be necessary for nuclear processing and/or transport to the cytoplasm (7). The failure of cordycepin to substantially inhibit enzyme synthesis could be attributed to an inability to reach a sufficient intracellular concentration of the drug or its active derivative(s) in vivo.

In conclusion, the data presented suggest that, by 2 hr after a single application of an active promoter and before the general increase in total RNA and protein synthesis (2), there occurs in the mouse epidermis the synthesis of specific mRNAs that, following subsequent translation, result in greatly increased ornithine and S-Ado-Met decarboxylase activities. This represents one of the earliest specific biosynthetic events known to occur after promoter treatment and, analogous to other stimulated-to-proliferate systems, may be essential for the increased DNA synthesis and cell division beginning in the epidermis 18 to 24 hr after promoter application (2).

In order to understand the mechanism of promotion, we will need to know what other gene activation phenomena occur in response to promoters, how such changes in gene expression are accomplished, and which of these effects are essential to promotion. A comparison of the effects on gene expression of promoters as well as hyperplastic but essentially nonpromoting agents would be helpful in determining which effects of promoters are unique to promotion and which are related to the irritant, inflammatory, and hyperplastic properties of promoters. Such a study is in progress.

REFERENCES


Induction of the Polyamine-biosynthetic Enzymes in Mouse Epidermis by Tumor-promoting Agents

T. G. O'Brien, R. C. Simsiman and R. K. Boutwell


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