Inhibition of 12-O-Tetradecanoylphorbol-13-acetate Induction of Ornithine Decarboxylase Activity, DNA Synthesis, and Tumor Promotion in Mouse Skin by Ascorbic Acid and Ascorbyl Palmitate

Robert C. Smart,1 Mou-Tuan Huang, Zheng Tao Han,2 Michael C. Kaplan, Antonino Focella, and Allan H. Conney3

ABSTRACT

The effects of topically applied 12-O-tetradecanoylphorbol-13-acetate (TPA) on the level of ascorbic acid in the epidermis and the effects of topically applied ascorbic acid, ascorbyl palmitate (a synthetic lipophilic derivative of ascorbic acid), palmitic acid and sorbitan monopalmitate on TPA-induced epidermal ornithine decarboxylase activity, epidermal DNA synthesis, and the promotion of skin tumors were evaluated in female CD-1 mice. Topical application of 5 or 16 nmol of TPA resulted in a 45-50% decrease in the amount of ascorbic acid per mg protein in mouse epidermis at 5 h after TPA application. Large topical doses of ascorbic acid inhibited TPA-induced tumor promotion in mouse epidermis, but smaller doses were inactive. The topical application of relatively small doses of ascorbyl palmitate had a marked inhibitory effect on TPA-induced ornithine decarboxylase activity, DNA synthesis, and tumor promotion in mouse epidermis. Ascorbic acid, palmitic acid, and sorbitan monopalmitate were less effective than ascorbyl palmitate as inhibitors of tumor promotion. The topical application of 4 μmol of ascorbyl palmitate inhibited by 60-76% the induction of epidermal ornithine decarboxylase activity and DNA synthesis that occurred after a single topical application of 2 nmol of TPA whereas similar doses of ascorbic acid had no inhibitory effect. The topical application of 4 μmol of ascorbyl palmitate together with 5 nmol of TPA twice weekly for 20 weeks to previously initiated mice inhibited by 91% the number of tumors per mouse.

INTRODUCTION

Carcinogenesis in mouse skin is a multistep process that can be divided into at least two distinct stages, termed initiation and promotion (1-4). Although the mechanisms of tumor promotion are not known, some studies have suggested that reactive oxygen species such as superoxide anion may be involved (5-7). Superoxide dismutase is an important enzymatic defense for the removal of superoxide anion, and the biomimetic copper (II) (3,5-diisopropylsalicylate), which possesses superoxide dismutase activity, has been demonstrated to inhibit TPA-induced ornithine decarboxylase activity and tumor promotion in mouse skin (5). Induction of epidermal ornithine decarboxylase and epidermal DNA synthesis are biochemical parameters closely associated with tumor promotion (8, 9), and inhibition of the TPA-dependent induction of these parameters has been utilized to evaluate potential inhibitors of tumor promotion. A wide variety of compounds inhibit TPA-induced tumor promotion in mouse skin, and many of these compounds possess antioxidant or reactive oxygen scavenging activity. Butylated hydroxyanisole (10, 11), butylated hydroxytoluene (10), sodium selenite (12), α-tocopherol (13), gluthathione (13), copper (II) (3,5-diisopropylsalicylate) (5), quercetin (14), ethanol, and dimethyl sulfoxide (15) are examples of compounds that possess antioxidant or reactive oxygen scavenging activity and that inhibit tumor promotion and/or certain biochemical events associated with tumor promotion in mouse skin. Ascorbic acid is an efficient scavenger of superoxide anion and based on its rate constant for the reduction of superoxide and its cellular concentration, ascorbic acid is considered to be an important cellular chemical defense against superoxide (16).

Although ascorbic acid is a naturally occurring biological antioxidant, its effect on tumor promotion in mouse skin has not been extensively evaluated. In one study, ascorbic acid has been reported to have no effect on TPA-induced ornithine decarboxylase activity in mouse skin (11). In a second study, it was reported that topical treatment of initiated mice with ascorbic acid for the first 3 weeks of croton oil application inhibited skin tumor formation by 25% (12). In an additional study, ascorbic acid, butylated hydroxytoluene, α-tocopherol, and reduced glutathione were added together to a chow diet and fed to mice initiated with 3-methylcholanthrene and promoted with croton oil. Treatment of mice with this combination of antioxidants inhibited skin tumor formation (17). Dietary ascorbic acid has also been reported to inhibit the incidence of dermal neoplasms induced by UV light in hairless mice (18). In cell culture, ascorbic acid has been reported to suppress 3-methylcholanthrene-induced transformation of C3H10T/5' cells (19, 20). Although these studies suggest a protective effect of ascorbic acid in cell transformation and in tumor promotion, it should also be pointed out that very high doses of ascorbic acid can promote bladder tumors in rats previously initiated with N-butyl-N-(4-hydroxybutyl)nitrosamine (21). In the present study, we have investigated the effect of ascorbic acid and its synthetic lipophilic antioxidant derivative, ascorbyl palmitate (see Fig. 1 for structures), on the induction by TPA of epidermal ornithine decarboxylase activity, DNA synthesis, and tumor promotion. Several compounds with structural features similar to ascorbyl palmitate have also been evaluated for their effect on tumor promotion and/or certain biochemical parameters associated with tumor promotion in mouse skin. In addition, we have examined the effect of topical application of TPA on the concentration of ascorbic acid in mouse epidermis.

MATERIALS AND METHODS

Materials. TPA was purchased from CRC, Inc., Chanhassen, MN, and 2,9-1,2-didecanoylglycerol was purchased from Avanti Polar Lipids, Birmingham, AL. [14C]Ornithine and [14H]Thymidine were purchased from Amersham Corp., Arlington Heights, IL. Pyrex 9-well glass plates, glass cover plates, and GF/A filters were purchased from Ace
Ascorbic Acid in the Epidermis. The topical application of TPA to mouse skin resulted in a decrease in the endogenous level of ascorbic acid in the epidermis. As shown in Fig. 2, the application of 16 nmol of TPA to mouse skin decreased the amount of ascorbic acid per mg protein by 20-30% at 1-3 h after its application and by 45-50% at 5-10 h. The level of epidermal ascorbic acid returned to control values by 18 h. A 45% decrease in the level of ascorbic acid in the epidermis was also observed.

RESULTS

Effect of Topical Application of TPA on the Concentration of Ascorbic Acid in the Epidermis. The topical application of TPA to mouse skin resulted in a decrease in the endogenous level of ascorbic acid in the epidermis. As shown in Fig. 2, the application of 16 nmol of TPA to mouse skin decreased the amount of ascorbic acid per mg protein by 20-30% at 1-3 h after its application and by 45-50% at 5-10 h. The level of epidermal ascorbic acid returned to control values by 18 h. A 45% decrease in the level of ascorbic acid in the epidermis was also observed.

Fig. 2. Effect of topical application of TPA on the concentration of ascorbic acid in mouse epidermis. TPA (16 nmol) in 200 µl acetone was applied to mouse skin. Control mice received acetone alone. The mice were killed at various times after the application of TPA, and the epidermis was removed. Epidermal ascorbic acid was quantitated as described in "Materials and Methods." The average concentration of ascorbic acid in the epidermis of acetone treated mice was 4.99 ± 0.29 nmol of ascorbic acid per mg epidermal protein. Values, mean ± SE from five mice.

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ASCORBIC ACID

ASCORBYL PALMITATE

DEHYDROASCORBYL PALMITATE

ISOASCORBYL PALMITATE

Fig. 1. Structures of ascorbic acid, ascorbyl palmitate, dehydroascorbic palmitate, and isoascorbic palmitate.
at 5 h after the topical application of 5 nmol of TPA to mouse skin (data not shown).

Effects of Ascorbic Acid on TPA Induction of Epidermal Ornithine Decarboxylase Activity, DNA Synthesis, and Tumor Formation. The effects of topically applied ascorbic acid on TPA-induced ornithine decarboxylase activity, DNA synthesis, and tumor formation in mouse epidermis were examined. As shown in Table 1, the topical application of 28 µmol ascorbic acid simultaneously with 2 nmol TPA inhibited TPA-induced epidermal ornithine decarboxylase activity by 35%, but lower doses of ascorbic acid had no inhibitory effect. The data in Table 2 suggest that the topical application of low doses of ascorbic acid (1.5–5.6 µmol) may enhance the TPA-dependent stimulation of thymidine incorporation into DNA whereas a higher dose of ascorbic acid (28 µmol) had no effect on the TPA-dependent stimulation of epidermal DNA synthesis. As shown in Fig. 3, the application of 6 or 28 µmol ascorbic acid simultaneously with 2 nmol TPA twice weekly for 21 weeks to mice previously initiated with DMBA resulted in a 39 and 76% decrease, respectively, in the average number of tumors per mouse, and the numbers of tumor-bearing mice were decreased by 3 and 86%, respectively, after 21 weeks of promotion. These results indicate that large topical doses of ascorbic acid can inhibit TPA-induced tumor promotion on mouse skin. Ascorbic acid is a water-soluble, polar molecule, that may not be efficiently absorbed into epidermal cells after topical administration, and this may account for the large dose of ascorbic acid that is required to inhibit tumor promotion and the biochemical events associated with it. Because of this possibility, we have studied ascorbyl palmitate, a synthetic lipophilic derivative of ascorbic acid.

Table 1. Effect of various topical doses of ascorbic acid and ascorbyl palmitate on TPA-induced DNA synthesis in mouse epidermis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]Thymidine incorporation into DNA (dpm/µg DNA)</th>
<th>% Inhibition of TPA-induced DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent control</td>
<td>24.2 ± 1.3 (44)</td>
<td></td>
</tr>
<tr>
<td>TPA (2 nmol)</td>
<td>89.6 ± 4.9 (44)</td>
<td></td>
</tr>
<tr>
<td>TPA (2 nmol) + ascorbic acid (1.5 µmol)</td>
<td>103.7 ± 24.2 (8)</td>
<td></td>
</tr>
<tr>
<td>TPA (2 nmol) + ascorbic acid (5.6 µmol)</td>
<td>120.4 ± 13.6 (8)*</td>
<td></td>
</tr>
<tr>
<td>TPA (2 nmol) + ascorbic acid (28 µmol)</td>
<td>89.3 ± 10.1 (8)</td>
<td></td>
</tr>
<tr>
<td>TPA (2 nmol) + ascorbyl palmitate (0.075 µmol)</td>
<td>79.2 ± 9.3 (12)</td>
<td>16</td>
</tr>
<tr>
<td>TPA (2 nmol) + ascorbyl palmitate (0.15 µmol)</td>
<td>67.6 ± 6.1 (12)*</td>
<td>34</td>
</tr>
<tr>
<td>TPA (2 nmol) + ascorbyl palmitate (0.30 µmol)</td>
<td>67.7 ± 5.0 (16)*</td>
<td>34</td>
</tr>
<tr>
<td>TPA (2 nmol) + ascorbyl palmitate (0.75 µmol)</td>
<td>59.8 ± 5.3 (20)*</td>
<td>46</td>
</tr>
<tr>
<td>TPA (2 nmol) + ascorbyl palmitate (1.5 µmol)</td>
<td>54.0 ± 4.3 (35)*</td>
<td>54</td>
</tr>
<tr>
<td>TPA (2 nmol) + ascorbyl palmitate (4.0 µmol)</td>
<td>49.5 ± 4.0 (16)*</td>
<td>61</td>
</tr>
</tbody>
</table>

* Statistically different (P < 0.05) from TPA alone as determined by Student’s t test.
TPA-induction of ornithine decarboxylase activity after a 28 μmol dose of ascorbic acid. As shown in Table 2, ascorbyl palmitate is also an effective inhibitor of TPA-induced epidermal DNA synthesis. The topical application of only 0.15 μmol ascorbyl palmitate simultaneously with 2 nmol TPA inhibited TPA-induced DNA synthesis by 34% compared to the lack of an inhibitory effect of a 1.5 to 28 μmol topical dose of ascorbic acid. These results indicate that ascorbyl palmitate is at least 30-fold more effective than ascorbic acid as an inhibitor of some of the biochemical parameters associated with tumor promotion in mouse skin. Topical application of certain diacylglycerols can mimic the effect of TPA in mouse skin with regard to the induction of ornithine decarboxylase activity and DNA synthesis (25). As shown in Table 1 (experiment 3), ascorbyl palmitate (4 μmol) inhibited sn-1,2-didecanoylglycerol-induced ornithine decarboxylase activity by 42%.

The effect of ascorbyl palmitate on TPA-induced tumor promotion was also evaluated. Topical application of 0.8 or 4 μmol ascorbyl palmitate simultaneously with 5 nmol of TPA twice weekly to the dorsal shaven region of DMBA-initiated CD-1 mice for 20 weeks markedly inhibited the average number of tumors per mouse as well as the percentage of tumor-bearing mice (Fig. 4, A and B). As shown in Fig. 4, topical application of 0.16, 0.8, and 4 μmol ascorbyl palmitate simultaneously with 5 nmol TPA inhibited the average number of tumors per mouse at 20 weeks by 16, 46, or 91%, respectively, and the percentage of tumor-bearing mice was decreased by 5, 18, or 76%, respectively. In another similar tumor study with DMBA-initiated mice, 4 μmol ascorbyl palmitate was applied topically 1 h before 5 nmol TPA, twice weekly for 20 weeks. At this time, ascorbyl palmitate treatment had decreased the average number of tumors per mouse by greater than 90% (data not shown). The effects of ascorbyl palmitate and ascorbic acid on TPA-induced tumor formation are compared in Fig. 4, C and D. Ascorbyl palmitate (0.8 μmol) inhibited the average number of TPA-induced tumors per mouse by 46% while the same dose of ascorbic acid had no inhibitory effect. These results demonstrate that ascorbyl palmitate is a much more potent inhibitor of TPA-induced tumor promotion on mouse skin than ascorbic acid. In order to better understand the structural properties of ascorbyl palmitate that are responsible for its biological effects, we studied the effects of some structurally related compounds on the TPA-dependent induction of epidermal ornithine decarboxylase activity.

Although the application of 4 μmol ascorbyl palmitate to mouse skin markedly inhibited the induction of ornithine decarboxylase activity and DNA synthesis by 2 nmol of TPA little or no inhibitory effect was observed after the topical application of up to 10 μmol of ascorbic acid (Tables 1 and 2) or up to 4 μmol of palmitic acid (Tables 3 and 4). Interestingly, the topical application of 4 μmol of dehydroascorbyl palmitate, isoaescorbil palmitate, or sorbitan monopalmitate (amphipathic substances that resemble ascorbyl palmitate by possessing polar sugar and a hydrophobic fatty acid ester) markedly inhibited the induction of ornithine decarboxylase activity by 2 nmol of TPA (Table 3). However, studies with palmitic acid ethyl ester indicated that the topical application of 4 μmol of the compound together with 5 nmol of TPA resulted in a 140%
The percentage of tumor-bearing mice was inhibited by 32%. A
and radioactivity in epidermal DNA was determined. Data, mean ± SE. Numbers
carboxylase activity (data not shown).

Application of palmitic acid (4 μmol) along with 5 nmol TPA (Tables 3 and 4), this compound did inhibit the tumor-promot
induction by TPA of ornithine decarboxylase or DNA synthesis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ornithine decarboxylase activity (μmol 14CO2 released/ mg protein/h)</th>
<th>% Inhibition of TPA-induced ornithine decarboxylase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cetone</td>
<td>42 ± 6</td>
<td></td>
</tr>
<tr>
<td>PA (2 nmol)</td>
<td>970 ± 122</td>
<td>69</td>
</tr>
<tr>
<td>PA (2 nmol) + ascorbyl palmitate (4 μmol)</td>
<td>326 ± 86</td>
<td>69</td>
</tr>
<tr>
<td>PA (2 nmol) + dehydroascorbyl palmitate (4 μmol)</td>
<td>443 ± 116</td>
<td>57</td>
</tr>
<tr>
<td>PA (2 nmol) + isoascorbyl palmitate (4 μmol)</td>
<td>431 ± 39</td>
<td>58</td>
</tr>
<tr>
<td>PA (2 nmol) + sorbitan monopalmitate (4 μmol)</td>
<td>651 ± 76</td>
<td>34</td>
</tr>
<tr>
<td>PA (2 nmol) + palmitic acid (4 μmol)</td>
<td>1147 ± 192</td>
<td>34</td>
</tr>
<tr>
<td>PA (2 nmol) + trans-retinoic acid (2 nmol)</td>
<td>111 ± 43</td>
<td>93</td>
</tr>
</tbody>
</table>

* Statistically different (P < 0.05) from TPA alone as determined by Student’s t test.

Comparison of the effect of ascorbyl palmitate, sorbitan monopalmitate, palmitic acid ethyl ester, and palmitic acid on TPA-induced DNA synthesis in mouse epidermis

TPA (2 nmol) was applied with or without test compound in 200 μl of acetone. Controls received acetone alone. Eighteen h later the mice were injected with [3H] thymidine. Forty min later the mice were sacrificed, the epidermis was removed, and radioactivity in epidermal DNA was determined. Data, mean ± SE. Numbers in parentheses in the second column, number of mice used.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]Thymidine incorporation into DNA (dpm/μg DNA)</th>
<th>% Inhibition of TPA-induced DNA synthesis</th>
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<tbody>
<tr>
<td>cetone</td>
<td>24.2 ± 1.3 (44)</td>
<td>54</td>
</tr>
<tr>
<td>PA (2 nmol)</td>
<td>89.6 ± 4.9 (44)</td>
<td>54</td>
</tr>
<tr>
<td>PA (2 nmol) + ascorbyl palmitate (1.5 μmol)</td>
<td>54.0 ± 4.3 (35)*</td>
<td>54</td>
</tr>
<tr>
<td>PA (2 nmol) + sorbitan monopalmitate (1.5 μmol)</td>
<td>61.6 ± 6.8 (8)*</td>
<td>43</td>
</tr>
<tr>
<td>PA (2 nmol) + palmitic acid ethyl ester (1.5 μmol)</td>
<td>69.9 ± 7.3 (16)*</td>
<td>30</td>
</tr>
<tr>
<td>PA (2 nmol) + palmitic acid (1.5 μmol)</td>
<td>91.6 ± 6.0 (8)</td>
<td>43</td>
</tr>
<tr>
<td>PA (2 nmol) + palmitic acid (4.0 μmol)</td>
<td>81.0 ± 5.8 (8)</td>
<td>13</td>
</tr>
</tbody>
</table>

* Statistically different (P < 0.05) from TPA alone as determined by Student's t test.

Comparison of the effect of an equilibrium 4 μmol dose of stimulation in the TPA-dependent induction of ornithine de

More detailed studies with sorbitan monopalmitate revealed that this substance inhibited TPA-induced DNA synthesis and tumor promotion on mouse skin (Table 4 and Fig. 4). Twice weekly topical application of various doses of sorbitan monopalmitate simultaneously with 5 nmol of TPA to DMBA-induced mice inhibited tumor formation, and at the highest dose of sorbitan monopalmitate examined (4 μmol), the average number of tumors per mouse was decreased by 74% and the percentage of tumor-bearing mice was decreased by 30% (Fig. 4, E and F). Sorbitan monopalmitate was not as effective as ascorbyl palmitate at inhibiting tumor promotion by TPA.

Although palmitic acid was not an effective inhibitor of the induction by TPA of ornithine decarboxylase or DNA synthesis (Tables 3 and 4), this compound did inhibit the tumor-promoting effect of TPA (Fig. 4, G and H). Twice weekly topical application of palmitic acid (4 μmol) along with 5 nmol TPA inhibited the average number of tumors per mouse by 53%, and the percentage of tumor-bearing mice was inhibited by 32%. A

ascorbic acid per mg protein is decreased by the application of TPA to mouse skin and that simultaneous application of large doses of ascorbic acid along with TPA can inhibit tumor promotion on mouse skin. Application of TPA to mouse skin is thought to increase the production of active oxygen (5-7), and this effect may result in the depletion of epidermal ascorbic acid that is described in Fig. 2. In addition, earlier studies have shown that application of TPA to mouse skin increases protein synthesis so that a portion of the decrease in the amount of ascorbate per mg protein may be caused by an increased amount of epidermal protein. Ascorbic acid is an important biological antioxidant, and it has been suggested that ascorbic acid is a principal chemical defense against superoxide anion (16). It has also been postulated that the generation of reactive oxygen species such as superoxide anion may be involved in TPA-induced tumor promotion on mouse skin (5-7). Our results suggest that epidermal ascorbic acid may play a role in preventing TPA-induced tumor promotion. Other studies have indicated that ascorbic acid is depleted during inflammation, but the relationship (if any) between inflammation, tumor promotion, and ascorbic acid is not known. Although the mechanism of the inhibition of TPA-induced tumor promotion on mouse skin by ascorbic acid is not known, it may be related to the antioxidant activity of ascorbic acid or to its reported ability to inhibit the binding of TPA to its receptor (28).

We found that very large doses of ascorbic acid were required to inhibit TPA-induced tumor promotion, and we studied the ability of ascorbyl palmitate to act as an antipromoter since it is lipophilic and possesses antioxidant activity. The results of these experiments demonstrate that ascorbyl palmitate is a much more effective inhibitor of TPA-induced ornithine decarboxylase induction, epidermal DNA synthesis, and tumor promotion than ascorbic acid. Ascorbyl palmitate retains the antioxidant activity associated with ascorbic acid, but unlike ascorbic acid, it is an amphipathic molecule due to its polar ascorbic acid head and long hydrophobic palmitic acid side chain. We found that other amphipathic substances related to ascorbyl palmitate, such as sorbitan monopalmitate, isoascorbyl palmitate, and dehydroascorbyl palmitate, also inhibit tumor promotion and/or biochemical parameters associated with tumor promotion, and palmitic acid is also active. Since sorbitan monopalmitate, palmitic acid, and dehydroascorbyl palmitate do not have antioxidant activity, the results suggest that the antioxidant activity of ascorbyl palmitate may not be necessary for its antipromoter activity.

Protein kinase C activity and the receptor for TPA copurify, and protein kinase C activity is stimulated by TPA in vitro (29). It has been suggested that the activation of protein kinase C by tumor promoters is a critical event in tumor promotion (30, 31). In the membrane, protein kinase C forms a catalytically active complex with phospholipids and Ca2+, and the addition of TPA stimulates protein kinase C activity (30, 31). Local anesthetics and other phospholipid interacting compounds such as chlorpromazine have been shown to inhibit this complex...
formation (32). Several phospholipid-interacting compounds are capable of inhibiting the binding of TPA to its receptor and also the subsequent activation of protein kinase C (32). These inhibitors of TPA binding are thought to cause inhibitory effects through perturbation of the membrane ultrastructure. Likewise, the compounds examined in the present study may be inhibiting tumor promotion by their interaction with and perturbation of the cell membrane which could disrupt the formation of a catalytically active protein kinase C complex. Preliminary results indicate that ascorbyl palmitate is an inhibitor of TPA-stimulated protein kinase C activity.\(^5\) sn-1,2-Diacylglycerols have been shown to activate protein kinase C activity in an analogous fashion to TPA (33) and also to mimic the effects of TPA on the biochemical parameters associated with tumor promotion in mouse skin in vivo (25). Additional studies have shown that ascorbyl palmitate can inhibit sn-1,2-diacylglycerol-induced ornithine decarboxylase activity in mouse skin (Table 1).

In summary, the results presented in this study demonstrate that topical application of TPA produces a decrease in the level of ascorbic acid in the epidermis and that large topical doses of ascorbic acid can inhibit TPA-induced tumor promotion in mouse skin. We found that ascorbyl palmitate, a synthetic lipophilic derivative of ascorbic acid, is much more potent than ascorbic acid as an inhibitor of tumor promotion by TPA. The inhibitory effects of ascorbyl palmitate on the TPA-dependent induction of ornithine decarboxylase activity, DNA synthesis and/or tumor promotion may be unrelated to the antioxidant activity of ascorbyl palmitate.

REFERENCES


\(^5\) Unpublished observations.
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