Inhibition of Benzoil Peroxide-Mediated Tumor Promotion in 7,12-Dimethylbenz(a)anthracene-Initiated Skin of Sencar Mice by Antioxidants Nordihydroguaiaretic Acid and Diallyl Sulfide

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Benzoyl peroxide (BPO), a free radical generating compound, is widely used in topical medications prescribed for acne vulgaris and in cosmetic products. It has been shown to possess tumor-promoting activity in murine skin initiated with chemical carcinogens such as 7,12-dimethylbenz(a)anthracene (DMBA). In the present study we assessed the effect of the antioxidants nordihydroguaiaretic acid (NDGA) and diallyl sulfide (DAS) against BPO-mediated tumor promotion in murine skin. Pretreatment of Sencar mice with NDGA and DAS prior to skin application of BPO resulted in a time- and dose-dependent inhibition of epidermal ODC induction caused by BPO. Tumor initiation was achieved by a single topical application of DMBA (10 μg/animal) to Sencar mice. Ten days later tumor promotion was begun by twice-weekly topical application of BPO (20 mg/animal).

The anticarcinogenic effects of NDGA (25 μmol/mouse) and DAS (20 μmol/mouse) were evaluated by administering these agents topically 60 min prior to each BPO application. After 26 weeks on test, the number of benign papillomas/mouse were 0.10 ± 0.07 and 2.15 ± 0.30 in the NDGA and DAS pretreated group of animals as compared to 4.40 ± 1.14 in animals receiving BPO alone. After 51 weeks on test, the number of squamous cell carcinomas/mouse were 0.00 ± 0.00, 0.35 ± 0.10 in the NDGA and DAS pretreated group of animals as compared to 0.65 ± 0.12 in animals receiving BPO alone. From these data we suggest that the antioxidants NDGA and DAS can abrogate the tumor-promoting effects of BPO in murine skin and that NDGA is substantially more effective than DAS in this regard. J Invest Dermatol 94:162-165, 1990

Benzoyl peroxide (BPO), a free radical generating compound and a strong oxidizing agent, is widely used in industry as a polymerization initiator [1], as a bleaching agent for flour and cheese [2], and as an additive in cosmetics and pharmaceuticals, especially those employed for the treatment of acne vulgaris [2,3]. In murine skin, BPO has been shown to be a moderately effective tumor promoter [4] in animals treated with the chemical carcinogens 7,12-dimethylbenz(a)anthracene (DMBA), benzo(a)pyrene, and N-methyl-N-nitro-N-nitrosoguanidine. O'Connell et al [5] have shown that repetitive application of BPO to mouse skin papillomas increases malignant progression of these otherwise benign tumors. BPO is known to be an irritant for human skin [6]. In view of these observations some concern has been expressed regarding the frequent use of BPO by humans [4-6]. However, because of its wider applicability and its unavoidable exposure to humans, there is a need to define such compounds, which may be effective in abrogating the tumorigenic effects of BPO. In the present study we demonstrate that the plant products nordihydroguaiaretic acid (NDGA) and diallyl sulfide (DAS) afford protection against BPO-induced tumor promotion and subsequent progression in DMBA-initiated skin of Sencar mice. This effect may be due to inhibition of the induction of epidermal ornithine decarboxylase (ODC) caused by BPO.

MATERIALS AND METHODS

Chemicals DMBA, BPO, DAS (>97% pure), NDGA (>97% pure) (Aldrich Chemical Co., Milwaukee, WI), TPA, L-ornithine, and pyridoxal 5'-phosphate (Sigma Chemical Co., St. Louis, MO) were purchased in the purest form commercially available. Ornithine, DL-[1-14C] (50.3 mCi/mmol) was obtained from New England Nuclear, Chicago, IL.

Inhibition of ODC Induction Six-week-old female Sencar mice obtained from the NCI-Frederick Cancer Research Facility, Frederick MD, were used. Mice at the age of eight weeks were shaved with electric clippers 2 d prior to treatment. BPO (20 mg/mouse) was applied topically to the shaved skin in 0.2 ml acetone and the animals were killed at desired time after treatment. In experiments where the effect of DAS (20 μmol/0.2 ml/mouse) or NDGA (25
μmol/0.2 ml/mouse) was evaluated, the compounds were applied topically 60 min prior to the application of BPO. Control mice received acetone alone and six animals were utilized in each group. The animals were killed by cervical dislocation and the dorsally shaved skin was removed and epidermal homogenate prepared as described previously [7]. ODC activity was determined in 100,000 X g supernatants by the method of O'Brien et al [8] as described by Verma et al [9]. Enzyme activity was expressed as pmol CO₂ released/h/mg protein. Protein content was determined by the method of Bradford [10].

**Treatment of Animals for Tumor Studies** Mice at eight weeks of age were shaved on dorsal skin with electric clippers two days prior to the beginning of the experiment. Only animals in the resting phase of the hair cycle were used. Skin tumors were chemically induced by a standard two-stage initiation-promotion protocol as described previously [11]. Initiation was accomplished with a single topical application of 10 μg of DMBA under subdure light. Ten days following initiation, all of the mice were treated twice weekly with topical application of 20 mg of BPO for 26 weeks. For the antitumor promotion studies, NDGA (25 μmol/0.2 ml acetone/mouse) or DAS (20 μmol/0.2 ml acetone/mouse) was applied topically 60 min prior to each application of BPO as shown in Figure 1. Mice treated with acetone alone, DMBA alone, NDGA alone, DAS alone, or BPO alone served as negative controls and yielded no tumors (data not shown). The regimens of dose selection for BPO was based on prior published work [4] and the doses of NDGA and DAS were based on our unpublished observations and on the data obtained in the present study for the inhibition of induction of ODC activity. The detailed description of each treatment is provided in the legends of the individual figures and tables. Treatment was continued until the termination of the experiment. The number of suspected papillomas and carcinomas were counted weekly and were verified histologically at the time when tumorbearing mice died or at the conclusion of the experiment. The statistical significance of difference between two groups was evaluated using the Student t test at each time point. The criteria for the diagnoses of various tumors was the same as described by O'Connell et al [5] and from our laboratory [12]. In brief, papillomas are cauliflower-like exophytic tumors with a narrow or broad base consisting of a series of connective tissue folds covered by a stratified squamous epithelium usually without cellular atypia; keratoacanthomas are cup- or dome-shaped neoplasms with a central crater filled with keratin and lip-shaped borders composed of hyperplastic epidermal overgrowth (in central region, numerous irregular and sometimes atypical epithelial proliferations extending into the dermis); and squamous cell carcinomas are composed of very irregular endophytic epithelial growths with numerous cellular atypias. They were usually very differentiated with formation of keratinized layers and horny pearls. For this study, keratoacanthomas and squamous cell carcinomas are grouped as carcinomas.

**RESULTS AND DISCUSSION**

The data shown in Fig 2 indicate that a single topical application of BPO to Sencar mice resulted in a time-dependent induction of ODC activity. Maximum induction was observed 16 h after BPO application, and the inhibition of BPO-Mediated Tumor Production

**Figure 1.** Experimental protocol for evaluation of antitumor promoting effects of NDGA and DAS.

**Figure 2.** Animals received a single topical application of NDGA (25 μmol/mouse) or DAS (20 μmol/mouse) 60 min before BPO application (20 mg/mouse). The animals were killed at desired time and epidermal ODC activity determined as described in Materials and Methods. Values represent mean ± SEM of 6 animals.

**Figure 3.** Three groups of Sencar mice (20 in each group) were initiated with DMBA (10 μg) and promoted with BPO (20 mg/mouse). For the antitumor-promotion studies, mice were pretreated with acetone (open circles) or NDGA (solid circles) or DAS (solid triangles) 60 min prior to each application of BPO. The number of tumors per mouse (top panel) and the percent of mice with tumors were plotted as a function of weeks on test. Treatment and other details are provided in Figure 1 and text.
Table I. Protection by NDGA and DAS Against Skin Papilloma and Carcinoma Formation in DMBA-Initiated and BPO-Promoted Sencar Mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Number of mice at risk</th>
<th>Papillomas/mouse (26 weeks)</th>
<th>Inhibition (%)</th>
<th>Carcinomas/mouse (51 weeks)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA alone</td>
<td>20</td>
<td>NTF</td>
<td></td>
<td>NTF</td>
<td></td>
</tr>
<tr>
<td>BPO alone</td>
<td>20</td>
<td>NTF</td>
<td></td>
<td>NTF</td>
<td></td>
</tr>
<tr>
<td>DMBA + BPO</td>
<td>20</td>
<td>4.40 ± 1.14</td>
<td>0.65 ± 0.12</td>
<td>0.65 ± 0.12</td>
<td>31</td>
</tr>
<tr>
<td>DMBA + DAS + BPO</td>
<td>20</td>
<td>2.15 ± 0.36</td>
<td>0.65 ± 0.10</td>
<td>0.65 ± 0.10</td>
<td>100</td>
</tr>
<tr>
<td>DMBA + NDGA + BPO</td>
<td>20</td>
<td>0.10 ± 0.07</td>
<td>0.65 ± 0.10</td>
<td>0.65 ± 0.10</td>
<td>100</td>
</tr>
</tbody>
</table>

* Each value represents mean ± SEM of 20 animals.
† Female Sencar mice were initiated with a single topical application of DMBA (10 μg/0.2 ml acetone/mouse). Ten days later, BPO (20 mg/0.2 ml/mouse) was applied topically twice weekly for 26 weeks. NDGA and DAS were applied topically 1 h before each BPO application. The number of papilloma reported here represents the gross yield/mouse after 26 weeks of tumor promotion while number of carcinoma represents the gross yield/mouse after 51 weeks, at which time the experiment was terminated. All the applications were stopped after 26 weeks of tumor promotion.
‡ NT = no tumor formed.
§ *p < 0.05, Student's t test.

Treatment. Treatment of animals with topically applied NDGA and DAS prior to BPO application resulted in significant inhibition (up to 60%) of the enzyme induction caused by BPO. Direct addition of BPO or NDGA to the ODC incubation mixture produced negligible or no effect on ODC activity (data not shown) indicating that the inhibition of ODC induction is not related to residual NDGA or DAS which may be present in epidermal cytosolic preparations. NDGA is a potent inhibitor of the lipoygenase pathway of arachidonic acid metabolism and is known to inhibit TPA induced ODC induction [13, 14]. The inhibitory effect of NDGA on BPO-mediated ODC induction suggests that BPO may mimic the TPA effect on murine tumor promotion. Pretreatment of animals with DAS also showed inhibition of BPO-induced ODC activity, although this was much less pronounced as compared to that of NDGA (Fig 2).

The effect of pretreatment of animals with NDGA and DAS on BPO-mediated tumor promotion in DMBA-initiated Sencar mice is shown in Fig 3. Pretreatment of animals with NDGA and DAS abrogated the tumor-promoting effect of BPO as evidenced by the decreased total number as well as the lower incidence of tumors observed in these animals. After 26 weeks on test, the number of benign papillomas/mouse were 0.10 ± 0.07 and 2.15 ± 0.30 in the NDGA and DAS pretreated groups, respectively, compared to 4.40 ± 1.14 in animals receiving BPO alone. After 51 weeks on test, the number of squamous cell carcinomas/mouse were 0.00 ± 0.00 and 0.35 ± 0.10 in the NDGA and DAS pretreated animals, respectively, compared to 0.65 ± 0.12 in animals receiving BPO alone (Table I). Similarly, when tumor data are considered as the cumulative number of papillomas or carcinomas, pretreatment of animals with NDGA and DAS afforded significant protection against skin tumorigenesis as compared to BPO treated animals alone. Consistent with the ODC inhibition data, NDGA was more effective than DAS in reducing both the number of papillomas and carcinomas.

Although little is known about the mechanism of tumor promotion by BPO, it does share certain characteristics with other potent tumor promoters such as inhibition of metabolic cooperation in cultured keratinocytes and CHO cells [4,15,16], and induction of ODC and protein kinase C in mouse skin and in skin keratinocytes [17,18]. The induction of ODC activity is considered to be closely associated with the tumor-promoting activity of a variety of tumor promoters [19]. It is involved in polyamine synthesis, which plays a major role in cell proliferation and differentiation [20,21]. The importance of ODC induction in tumor promotion is evident from the fact that several inhibitors of ODC are capable of inhibiting tumor promotion in murine skin [22]. NDGA and a variety of other compounds, among them quercetin, morin, n-propyl gallate, and other flavonoids and antioxidants which inhibit ODC induction, have also been found to be effective inhibitors of TPA-mediated tumor promotion [23,24]. Thus, the inhibition of BPO-induced ODC activity by NDGA and DAS may explain the inhibition of tumor promotion by these compounds. BPO is a tumor promoter that produces oxidative stress within target cells [25] and generates free radicals [16,26], our unpublished observation) in skin which is another possible explanation for tumor promotion. Our results showing inhibition of BPO-mediated tumor promotion by DAS and NDGA may also be due to the antioxidant and free radical scavenging actions of these compounds. By scavenging free radicals and inhibiting ODC induction and lipoygenase activities, NDGA may distort the growth regulatory and mitogenic functions of polynuclear and arachidonic acid metabolites.

In conclusion, our data indicate that the naturally occurring antioxidants DAS and NDGA can abrogate BPO-mediated tumor promotion in DMBA-initiated murine skin and NDGA was more effective than DAS in its anti-tumor promoting effects. Since NDGA appears to be nontoxic it could prove useful in diminishing the cancer risk associated with BPO.

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REFERENCES

9. Verma AK, Shapas BG, Rice HM, Bourwell RK: Correlation of the inhibition by retinoids of tumor promoter-induced mouse epider-


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