Inhibition of 7,12-Dimethylbenz(a)anthracene-induced Rat Mammary Carcinogenesis by Concomitant or Postcarcinogen Antioxidant Exposure

David L. McCormick, Nancy Major, and Richard C. Moon

ABSTRACT

When administered prior to or at the time of carcinogen exposure, the phenolic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are effective inhibitors of carcinogenesis in several target organs. However, chronic, postcarcinogen administration of BHT apparently enhances tumorigenesis in certain animal models for liver and lung cancer. The present study was performed to determine the effects of BHA and BHT on mammary carcinogenesis when antioxidant exposure is limited to defined periods encompassing or following carcinogen availability. At 50 days of age (Time 0), virgin female Sprague-Dawley rats (25/group) were given a single intragastric dose of 8 mg of 7,12-dimethylbenz(a)anthracene. Basal diet (Wayne Lab Meal) was supplemented with 5000 or 2500 mg of BHA or BHT/kg by the following protocol: 2 weeks before until 1 week after carcinogen administration; 1 week after carcinogen administration until the end of the study; or none. The experiment was terminated 210 days after 7,12-dimethylbenz(a)anthracene administration, and all mammary tumors were confirmed histologically. When administered by the 2 weeks before to 1 week after schedule, both BHA and BHT were effective inhibitors of mammary carcinogenesis. However, the compounds also were active in chemoprevention when administered by the 1 week after to end protocol. These data indicate that the anticarcinogenic activity of antioxidants is not limited to influences on carcinogen metabolism, since both BHA and BHT inhibited mammary tumor induction when their administration was begun following clearance of the carcinogen from the mammary gland. The anticarcinogenic activity of postcarcinogen administration of BHA and BHT in the mammary gland is in contrast to the apparent tumor-enhancing activity of BHT in the liver and lung.

INTRODUCTION

The phenolic antioxidants BHA and BHT inhibit chemical carcinogenesis in several organ systems, including the lung (42), colon (46, 47), skin (33), liver (40), and mammary glands (16, 41), of experimental animals. In general, studies of antioxidant modification of carcinogenesis have involved administration of the compounds prior to or simultaneous with carcinogen exposure (22). For example, Wattenberg (41) reported a significant inhibition of mammary tumor induction in female Sprague-Dawley rats when a gavage dose of BHA or BHT was given 1 hr prior to treatment with DMBA. Similar anticarcinogenic activity in the DMBA/rat mammary carcinoma system and in other animal tumor models has been observed when antioxidants have been administered as a dietary supplement (7, 16). This inhibition of carcinogenesis is presumably mediated via alterations in carcinogen metabolism; BHA and BHT both have the capacity to modify the activity of enzymes involved in carcinogen metabolism (4, 10, 21, 23), and the anticarcinogenic activity of BHT appears to be limited to models using a carcinogen which requires metabolic activation (17).

Although the anticarcinogenic activity of antioxidants administered prior to or concomitant with carcinogen exposure is well documented, generalizations regarding the effects of postcarcinogen antioxidant administration are more difficult to draw. In studies using animal tumor models for the liver and lung, several groups have found that chronic postcarcinogen administration of BHT can increase ("promote") tumorigenesis in animals exposed to low doses of carcinogens (6, 30, 48). Furthermore, although studies using Wistar rats and B6C3F1 mice were negative, F344 rats fed 0.5 or 2.0% BHA in the diet for 2 years developed a dose-related incidence of proliferative and neoplastic lesions of the forestomach (12). On the basis of these data, it appears that chronic administration of BHA or BHT to experimental animals may result in complete carcinogenesis in susceptible animals and/or enhancement of tumorigenesis in tissues exposed previously to low doses of organotropic carcinogens.

By contrast, however, a number of biological actions of the phenolic antioxidants suggest that the compounds may inhibit rather than enhance carcinogenesis when administered subsequent to carcinogen exposure. BHA and BHT both inhibit the induction of ODC activity by the tumor promoter TPA, a trait shared with other antipromoting agents such as retinoic acid and indomethacin (20). BHA has significant antiinflammatory activity, perhaps related to influences on prostaglandin biosynthesis (36), and reports from 2 laboratories indicate that diets supplemented either with BHA (34) or a combination of BHT, α-tocopherol, L-ascorbate, and glutathione (5) can inhibit tumor promotion in mouse skin. These data suggest that, under appropriate conditions, antioxidants may have chemopreventive rather than enhancing activity in the postcarcinogen phase of tumor development.

In light of the documented anticarcinogenic activity of BHA and BHT when administered for a short period at the time of carcinogen treatment and the uncertain effects of chronic administration of these compounds following carcinogen exposure, the present study was conducted to determine the effects of different
schedules of antioxidant administration on rat mammary carcinogenesis induced by DMBA. In this study, BHA or BHT were administered in the diet either for a short period prior to and during the time of carcinogen availability or chronically following carcinogen exposure.

MATERIALS AND METHODS

Virgin female Sprague-Dawley rats were obtained as weanlings from Harlan/Sprague-Dawley, Indianapolis, IN. A total of 275 rats were used in the study. Animals were housed in groups of 3 in polycarbonate cages in a windowless room artificially illuminated on a 14-hr light/10-hr dark cycle and maintained at a temperature of 22 ± 1°C.

Basal diet for the study was Wayne Lab Blox MRH 22/5 (Allied Mills, Chicago, IL). This diet contains 22.0% protein and 5.0% fat by weight, and it contains no BHA, BHT, ethoxyquin, propyl gallate, or other synthetic antioxidants. As required by the protocol, a diet supplement of 5000 or 2500 mg of BHA or BHT (Sigma Chemical Co., St. Louis, MO) per kg of diet was added without vehicle to the basal diet. All animals had free access to diet and drinking water throughout the study, except for a 16-hr period prior to DMBA administration; during this period, rats had access to drinking water only.

At 36 days of age (Week -2), rats were randomized into groups by weight (see Table 1), and administration of experimental diets was begun. At 50 days of age (Day 0), all animals received a single intragastric dose of 8 mg of DMBA (Sigma) in 1 ml of sesame oil or sesame oil only. One week later (Week +1), experimental diets were changed, yielding 3 diet administration schedules: (a) controls, which received basal diet throughout the study; (b) -2 to +1 week antioxidant exposure, to determine the effects of BHA or BHT supplementation on the "early" phase of mammary carcinogenesis, consisting of metabolic activation of the carcinogen and its interaction with the mammary parenchymal cells; and (c) +1 week to end antioxidant exposure, to determine the effects of BHA and BHT on the "late" phase of mammary carcinogenesis, i.e., postcarcinogen tumor development. The choice of 1 week post-DMBA as the time for diet change was based on previous findings that BHA binding to mammary parenchymal cell DNA is complete, and remaining DHBA has been cleared from the mammary gland by 1 week postadministration (13, 17).

Beginning 4 weeks after DMBA administration, animals were palpated twice weekly to monitor mammary tumor appearance. Animals were observed daily and were weighed weekly throughout the study. Moribund animals were killed by CO2 asphyxiation; otherwise, all animals were killed 210 days after DMBA administration. Animals killed or found dead were necropsied promptly. Sections of all mammary tumors and any other grossly abnormal tissues were taken for histopathological classification. Livers were excised from all animals surviving until the end of the experiment and were weighed, and sections were taken for histological study. In addition, wholemounts were prepared from the abdominal-inguinal mammary glands of animals in Groups 1 to 5 (non-DMBA-treated controls) to determine possible influences of antioxidant administration on mammary gland differentiation and development. Tissues were fixed in 10% buffered formalin; sections were stained with hematoxylin and eosin, and wholemounts were stained with alcian carmine. Only histologically confirmed mammary tumors were used in the data analysis. Tumor pathology was defined according to the criteria of Young and Hallowes (50).

Inhibition of carcinogenesis was defined as a statistically significant reduction in cancer incidence, reduced carcinoma multiplicity, reduced total tumor multiplicity, and/or increased median cancer latent period in an antioxidant-treated group compared to the basal diet control group. Cancer incidence values were calculated by the life table method; statistical comparisons of incidence curves were made using the log-rank test (31). Intergroup comparisons of number of tumors per rat were performed via analysis of variance, based on square-root-transformed data as suggested by Snedecor and Cochran (35). Statistical comparisons of T50 were performed using the median test (19). Values for mean body weight and mean liver weight were compared using analysis of variance.

RESULTS

Mammary tumors were induced rapidly, without acute toxicity, in animals receiving a single intragastric dose of 8 mg of DMBA. Adenocarcinomas were characterized by acini of irregular size and shape, lined by multiple layers of well-differentiated epithelial cells. Connective tissue stroma was sparse. Papillary carcinomas consisted of irregular projections of connective tissue stroma bordered by multiple layers of epithelial cells. Papillary projections frequently contained regions of adenoscarcinoma. Adenomas consisted of large numbers of acini of normal size and shape lined by a single layer of epithelial cells, while fibroadenomas were largely acellular tumors consisting of connective tissue with small nests of acini of normal appearance. Approximately 25% of induced tumors were classified histologically as carcinomas; remaining tumors were predominantly adenomas or mixed tumors containing areas of adenoma and fibroadenoma. This high percentage of benign tumors in Sprague-Dawley rats receiving lower DMBA doses is consistent with previous reports (29). It should be noted, however, that many benign lesions were small, flat adenomas detectable only at autopsy; the ratio of palpable malignant to palpable benign tumors was approximately 1:2. Because of the high multiplicity of both benign and malignant mammary lesions, data is presented for the influence of antioxidants on both mammary carcinoma multiplicity and total tumor multiplicity.

Administration of BHA by both the -2 to +1 week and +1 week to end schedules reduced tumor multiplicity and increased tumor latent period compared to the basal diet control group. In terms of tumor multiplicity, the 2 treatment schedules were approximately equal in chemopreventive efficacy: both reduced tumor number by approximately 30% from control levels (Table 2). Similarly, administration of BHA by the -2 to +1 week and +1 week to end protocols yielded cancer latency curves which were significantly different from control but not from one another (Chart 1).

No clear relationship was observed between BHA dose and degree of cancer inhibition; total tumor number was similar in

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>DMBA dose (mg)</th>
<th>Diet supplement (mg/kg of diet)</th>
<th>Supplement administration period (wk)</th>
<th>Body wt (g)</th>
<th>Liver wt (g)</th>
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<tr>
<td>1</td>
<td>10</td>
<td>0</td>
<td>None</td>
<td>264 ± 0.43</td>
<td>11.3 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>5000</td>
<td>BHA -2 to +1</td>
<td>264 ± 0.43</td>
<td>11.2 ± 0.29</td>
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<tr>
<td>3</td>
<td>10</td>
<td>5000</td>
<td>BHT +1 to end</td>
<td>260 ± 0.5</td>
<td>11.5 ± 0.90</td>
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<td>4</td>
<td>10</td>
<td>5000</td>
<td>BHT -2 to +1</td>
<td>275 ± 0.7</td>
<td>13.4 ± 0.61</td>
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</tr>
<tr>
<td>5</td>
<td>10</td>
<td>5000</td>
<td>BHT -2 to +1</td>
<td>260 ± 0.5</td>
<td>11.5 ± 0.90</td>
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</tr>
<tr>
<td>6</td>
<td>10</td>
<td>5000</td>
<td>BHT +1 to end</td>
<td>273 ± 0.6</td>
<td>16.7 ± 0.20</td>
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</tr>
<tr>
<td>7</td>
<td>25</td>
<td>5000</td>
<td>BHT -2 to +1</td>
<td>285 ± 0.7</td>
<td>13.7 ± 0.46</td>
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</tr>
<tr>
<td>8</td>
<td>25</td>
<td>2500</td>
<td>BHT -2 to +1</td>
<td>281 ± 0.5</td>
<td>12.9 ± 0.52</td>
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</tr>
<tr>
<td>9</td>
<td>25</td>
<td>2500</td>
<td>BHT +1 to end</td>
<td>275 ± 0.6</td>
<td>14.5 ± 0.68</td>
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<tr>
<td>10</td>
<td>25</td>
<td>2500</td>
<td>BHT +1 to end</td>
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<td>15.0 ± 0.58</td>
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<tr>
<td>11</td>
<td>25</td>
<td>5000</td>
<td>BHT -2 to +1</td>
<td>274 ± 0.4</td>
<td>12.9 ± 0.63</td>
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<tr>
<td>12</td>
<td>25</td>
<td>5000</td>
<td>BHT +1 to end</td>
<td>293 ± 0.7</td>
<td>16.9 ± 0.36</td>
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<tr>
<td>13</td>
<td>25</td>
<td>5000</td>
<td>BHT +1 to end</td>
<td>284 ± 0.4</td>
<td>15.8 ± 0.40</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>25</td>
<td>2500</td>
<td>BHT +1 to end</td>
<td>284 ± 0.4</td>
<td>15.8 ± 0.40</td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± S.E.
b p < 0.01 versus relevant control group.
c p < 0.05 versus relevant control group.
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**Table 2**

<table>
<thead>
<tr>
<th>Diet group (mg of supplement/kg of diet)</th>
<th>T&lt;sub&gt;50&lt;/sub&gt;(days)</th>
<th>Cancer incidence (%)</th>
<th>Cancers/rat</th>
<th>Tumors/rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>88</td>
<td>2.24</td>
<td>8.60</td>
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<tr>
<td>5000 mg of BHA wk -2 to +1</td>
<td>156&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68</td>
<td>1.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.09&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>2500 mg of BHA wk -2 to +1</td>
<td>117</td>
<td>72</td>
<td>1.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5000 mg of BHA wk +1 to end</td>
<td>162&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.60&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.19&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2500 mg of BHA wk +1 to end</td>
<td>148&lt;sup&gt;e&lt;/sup&gt;</td>
<td>76</td>
<td>1.83&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.11&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>2500 mg of BHT wk -2 to +1</td>
<td>183&lt;sup&gt;c&lt;/sup&gt;</td>
<td>52&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.25&lt;sup&gt;f&lt;/sup&gt;</td>
<td>6.89&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>2500 mg of BHT wk +1 to +1</td>
<td>148&lt;sup&gt;g&lt;/sup&gt;</td>
<td>68</td>
<td>1.54&lt;sup&gt;g&lt;/sup&gt;</td>
<td>7.13&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>2500 mg of BHT wk +1 to end</td>
<td>131</td>
<td>72</td>
<td>1.56&lt;sup&gt;h&lt;/sup&gt;</td>
<td>4.07&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>2500 mg of BHT wk +1 to end</td>
<td>117</td>
<td>68</td>
<td>1.94&lt;sup&gt;i&lt;/sup&gt;</td>
<td>6.20&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.05 versus control.
<sup>b</sup> p < 0.10 versus control.
<sup>c</sup> p < 0.01 versus control.

This treatment protocol was no more effective than was the -2 to +1 week schedule in reducing carcinoma multiplicity (Table 2) and, indeed, it was somewhat less effective than was -2 to +1 week administration in increasing cancer latent period (Chart 2).

The inhibition of mammary carcinogenesis by BHT was more closely related to dose than was the activity of BHA. Decreases in carcinoma and total tumor multiplicity and increases in median cancer induction time were all greater in animals fed the high BHT dose than in rats receiving the lower-level supplement. Although differences between the high and low BHT doses were rarely of statistical significance, administration of the higher BHT dose was associated generally with a reduced tumor response and a higher level of statistical significance compared to control than was the low BHT dose (Table 2).

The inhibition of mammary carcinogenesis by BHA and BHT in the present study was achieved without general toxicity. As indicated in Table 1, no reduction in animal body weight gain was observed in groups receiving antioxidant supplementation by any protocol. The gross appearance and general health of animals fed BHA or BHT was good throughout the study. At necropsy, no consistent gross pathology was observed. However, chronic (+1 to end) dietary administration of BHT resulted in a statistically significant increase in liver weight in both DMBA- and sesame oil-treated rats. A similar, although smaller, influence of +1 to end administration of BHA was noted in oil controls. This increase in liver weight as a result of chronic BHT exposure is in agreement with previous studies (11, 30).

**DISCUSSION**

Dietary administration of BHA or BHT to DMBA-treated rats resulted in a statistically significant inhibition of mammary tumor induction in the present study. In general, the anticarcinogenic activity of BHA or BHT administered for a short period around the time of carcinogen exposure (-2 to +1 week) was equivalent to the activity of chronic, postcarcinogen (+1 week to end) treatment. The reduction in tumor multiplicity and the increase in latency in DMBA-treated rats.
An extensive literature has developed concerning the effects of antioxidant exposure on the metabolism of carcinogens and other xenobiotics. Antioxidants have been reported to inhibit mammary carcinogenesis in rats (39). A number of antioxidants inhibit prostaglandin synthesis in vivo and in vitro (9, 36). Indomethacin and flurbiprofen, both potent inhibitors of prostaglandin biosynthesis, have significant activity in inhibiting mammary tumor induction (3, 27, 28). Antioxidants also have the capacity to terminate free radical reactions; although the importance of such reactions in mammary carcinogenesis has not been demonstrated, the induction of activated oxygen species by tumor promoters and their inhibition by antipromoting agents is suggestive (15).

The results of the present study, when considered with previous reports of antioxidant modification of carcinogenesis, indicate that the tumor-inhibitory activity of postcarcinogen administration of BHT is target organ specific; although the compound inhibits tumor induction in the rat mammary gland, BHT appears to enhance tumor response in the rat liver and mouse lung. This differential activity may be due, at least in part, to the induction of BHT of a proliferative response in the liver and lung and the lack of such a response in the mammary gland. Administration of BHT by the +1 to end schedule resulted in a dose-related increase in rat liver weight in the present study (Table 1); this finding confirms previous reports (11, 30). Similarly, BHT administration increases rates of cell division in the lungs of treated mice, as indicated by increased incorporation of [3H]thymidine into DNA and increased lung weight (32, 49). By contrast, observation of mammary gland weight, however, was found in Groups 1 through 5 in the present study revealed no induction of mammary parenchymal growth by BHT or BHA; mammary glands from antioxidant-treated rats were indistinguishable from controls. The induction of cell proliferation by tumor promoters such as TPA and phenobarbital is well documented (1, 2, 30).

Thus, the induction of such a response by BHT in rat liver and mouse lung and the lack of BHT-induced proliferation in the mammary gland may at least partially explain the dramatically different effects of BHT on carcinogenesis in these organs.

Several other compounds, like BHA and BHT, have the capacity to inhibit both the early and late phases of mammary carcinogenesis. We have reported previously that –2 to +1 week and +1 week to end administration of retinyl acetate can inhibit the induction of mammary cancers in rats by DMBA (24) and by benzo(a)pyrene (25). Similarly, Wattenberg has reported inhibition of mammary carcinogenesis by benzyl isothiocyanate administered prior to (43) or after (44) DMBA exposure and by a dietary supplement of green coffee beans administered either from 15 days until 1 day prior to DMBA treatment or from +1 week to end (45).

These data indicate the anticarcinogenic activity of BHA and BHT in the DMBA rat mammary gland model system is not

The mechanism or mechanisms by which antioxidants inhibit the postcarcinogen phase of mammary carcinogenesis are unknown, although experiments performed in other organ systems suggest several possibilities. Dietary administration of BHA can inhibit the induction of ODC by TPA in mouse skin (20); other inhibitors of ODC induction, such as α-difluoromethylornithine, can inhibit mammary carcinogenesis in rats (39). A number of antioxidants inhibit prostaglandin synthesis in vivo and in vitro (9, 36). Indomethacin and flurbiprofen, both potent inhibitors of prostaglandin biosynthesis, have significant activity in inhibiting mammary tumor induction (3, 27, 28). Antioxidants also have the capacity to terminate free radical reactions; although the importance of such reactions in mammary carcinogenesis has not been demonstrated, the induction of activated oxygen species by tumor promoters and their inhibition by antipromoting agents is suggestive (15).

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These data indicate the anticarcinogenic activity of BHA and BHT in the DMBA rat mammary tumor model system is not...
limited to influences on carcinogen metabolism, since a nearly equivalent inhibition of carcinogenesis was obtained when BHA or BHT administration was begun after clearance of the DMBA from the mammary gland. Antioxidant administration has no apparent effect on the growth or development of the normal mammary gland. This lack of proliferative influence in the mammary gland may be the mechanistic basis for the differential effects of postcarcinogen administration of BHT on chemical carcinogenesis in the mammary gland as compared to the liver and lung. Conversely, however, the lack of effect of BHA and BHT on mammary differentiation excludes this as a possible mechanism by which these agents inhibit the postcarcinogen phase of mammary tumor development. The mechanism for the anticarcinogenic activity of antioxidants administered subsequent to carcinogen administration remains unknown.

REFERENCES


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