Different Modifying Response of Butylated Hydroxyanisole, Butylated Hydroxytoluene, and Other Antioxidants in N,N-Dibutylnitrosamine Esophagus and Forestomach Carcinogenesis of Rats

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ABSTRACT

The modifying effects of antioxidants were examined in a carcinogenesis system after N,N-dibutylnitrosamine treatment. Male F344 rats were given 0.05% N,N-dibutylnitrosamine in their drinking water for 4 wk and then treated with basal diet containing 2% butylated hydroxyanisole (BHA), 1% butylated hydroxytoluene (BHT) with 7 ppm vitamin K, 0.8% ethoxyquin, 5% sodium L-ascorbate, 5% sodium erythorbate, or no added chemical for 32 wk. BHA enhanced forestomach carcinogenesis but did not enhance esophageal carcinogenesis. BHT enhanced esophageal carcinogenesis but did not enhance forestomach carcinogenesis. Ethoxyquin significantly enhanced esophageal tumorigenesis. Neither esophageal nor forestomach carcinogenesis was affected by the other antioxidants evaluated. BHA significantly increased DNA synthesis of the forestomach epithelium, whereas BHT tended to increase that of the esophageal epithelium. Thus, BHA and BHT showed different modifying responses in carcinogenesis of the esophagus and forestomach.

INTRODUCTION

Treatment with antioxidants modifies the stage following chemical initiation in 2-stage carcinogenesis in many organs. For example, BHA (1), BHT (1), SA (2), SE (3), and ethoxyquin (3) promoted urinary bladder carcinogenesis. BHA inhibited liver carcinogenesis (1, 4), and low doses of BHA also promoted forestomach carcinogenesis (5). BHT also promoted thyroid carcinogenesis (6), but exhibited different results, such as promotion, inhibition, or no effect, for liver carcinogenesis (1, 7, 8). When BHT was given simultaneously with 2-fluorenylacetamide, it inhibited liver carcinogenesis but enhanced urinary bladder carcinogenesis (9).

DBN is carcinogenic for the esophagus, forestomach, liver, and bladder of rats. In the present study, we examined the modifying effects of BHA, BHT, ethoxyquin, SA, and SE in a carcinogenesis of DBN-initiated rats, especially in the esophagus and forestomach which are covered by a similar squamous epithelium.

MATERIALS AND METHODS

Animals. Six-wk-old male F344 rats (Charles River Japan, Inc., Atsugi, Japan) were used. The rats were housed 5 per plastic cage with wood chips (White Flake; Charles River Japan, Inc.) for bedding in an animal room with a 12-h light, 12-h dark cycle at 22 ± 2°C and 55 ± 10% relative humidity. Body weights, food consumption, and water intake were measured weekly up to wk 14, and every other wk from wk 16 to wk 36. The amounts of food and water consumed on 2 consecutive days of the week were measured on a per cage basis.

Chemicals. DBN was obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. BHA, BHT, and SA were food additive grade and were purchased from Wako Pure Chemical Ind., Osaka, Japan. Sodium erythorbate (food additive grade; Fujisawa Pharmaceutical Ind., Osaka, Japan) and ethoxyquin (Tokyo Kasei Kogyo Co., Ltd.) were also used in Experiment 1.

Experiment 1. Rats were randomly divided into 11 groups of 20 or 21 rats each. In the first 4 wk Groups 1 to 6 were given drinking water with 0.05% DBN in dark bottles. They were then given powdered basal diet (Oriental M; Oriental Yeast Co., Ltd., Tokyo, Japan) containing either 2% BHA (2 g/100-g diet) (Group 1), 1% BHT (with 7 ppm vitamin K in the drinking water, Group 2), 0.8% ethoxyquin (Group 3), 5% SA (Group 4), 5% SE (Group 5), or no added chemical (Group 6, control group) for 32 wk. Groups 7 to 11 were given drinking water without DBN for 4 wk and then powdered diet containing the corresponding test chemicals as in Groups 1 to 5, respectively. Doses of these test chemicals were chosen as the maximum tolerated doses after preliminary 13-wk toxicity studies (BHA and BHT) and 6-wk toxicity studies (ethoxyquin, SA, and SE). The rats were killed at the end of 36 wk.

The esophagus and stomach were removed together, inflated by intraluminal injection with 10% phosphate-buffered formalin solution for 5 min, slit along the greater curvature of the stomach, and then refixed in 10% phosphate-buffered formalin solution. The liver and kidney were weighed and placed in the same fixative. The bladder was also inflated by intraluminal injection of 10% phosphate-buffered formalin solution. These organs were examined histologically after embedding in paraffin and staining with hematoxylin-eosin.

Experiment 2. BHA or BHT was fed to rats as in Experiment 1. Control rats received basal diet without BHA or BHT. Three rats were killed 2 wk after the beginning of the experiment. Each rat received a single i.p. injection of BrdUrd (Sigma Chemical Co., St. Louis, MO) at a dose of 150 mg/kg of body weight 1 h before sacrifice. The bladder was fixed and processed as described above. The labeling index for BrdUrd was examined by an avidin-biotin-peroxidase complex method using an immunohistological technique with a monoclonal anti-BrdUrd antibody (Becton Dickinson Immunocytometry Systems, Mountain View, CA)(10).

Statistical Analysis. Data on incidences of lesions were analyzed for statistical significance with the χ² test. Other data were analyzed by using Student's t-test.

RESULTS

Experiment 1. Rats treated with antioxidants with or without DBN showed no toxic symptoms due to the chemicals, but the mean body weights of rats in Groups 1 to 5 given test chemicals were lower than that of the controls (Group 6) after wk 20. The final mean body weights and consumptions of food and water are summarized in Table 1. The final mean body weights in Groups 1 to 5 were less than that of Group 6. A trend toward slight reduction in the mean food intake was observed in Groups 2 to 5 when compared to Group 6.

Unpublished data.
Macroscopically, esophageal tumors were observed throughout in the esophagus of groups treated with DBN followed by test chemicals, especially BHT (Group 2) (Fig. 1). They were exophytic and were sometimes multiple, polypoid excrescences. Tumors of the forestomach were frequently observed in the area surrounding the limiting ridge in Group 1 treated with BHA. They were often multiple and occurred in the form of polyps.

Histological findings for the esophagus and forestomach are summarized in Table 2. Significant numbers of esophageal carcinomas occurred in Group 2 compared to Group 6. Of 9 cases of carcinomas in Group 2, 8 were noninvasive and 1 was invasive. The incidences of esophageal papilloma were significantly higher in each of Groups 2 and 3 compared to Group 6. There were no significant differences in esophageal epithelial hyperplasia among Groups 1 to 6. Treatment with DBN followed by BHA (Group 1) resulted in significantly higher forestomach carcinomas than treatment with DBN alone (Group 6) or BHA alone (Group 7). Moreover, no increase of glandular stomach lesions by BHA or BHT was apparent. The incidences of liver lesions in rats are summarized in Table 3. The incidences of hyperplastic foci, hyperplastic nodules, and hepatocellular carcinomas in Groups 1 to 5 were not significantly different from those of Group 6. Lesions of the bladder epithelium are summarized in Table 4. The incidences of PN hyperplasia, a putative preneoplastic lesion (11, 12), and the number of PN hyperplasia per 10-cm basement membrane in Groups 1 and 2 were significantly higher than those in Group 6. The incidence of papilloma in Group 2 was also significantly higher than that in Group 6. Inductions of PN hyperplasia, papilloma, and carcinoma in Groups 3 to 5 were not significantly different from those of Group 6.

Experiment 2. Labeling indices of the esophagus and forestomach epithelium in rats are summarized in Table 5. In the mucosa of the esophagus of rats treated with BHT, a trend toward increase in BrdUrd uptake was present when compared to the control group. In the hyperplastic mucosa of the esophagus of rats treated with BHT, a trend toward increase in BrdUrd uptake was present when compared to the control group.

**DISCUSSION**

BHA enhanced forestomach carcinogenesis of rats initiated by DBN, but did not modify esophageal carcinogenesis. In

Table 1 Average body weight and food consumption (wk 5 to 36) of rats in Experiment 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mean body wt (g)</th>
<th>Mean food consumption (g/rat/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DBN → BHA</td>
<td>127 ± 8</td>
<td>378 ± 13</td>
</tr>
<tr>
<td>2</td>
<td>DBN → BHT</td>
<td>130 ± 6</td>
<td>343 ± 23</td>
</tr>
<tr>
<td>3</td>
<td>DBN → Ethoxyquin</td>
<td>130 ± 7</td>
<td>353 ± 13</td>
</tr>
<tr>
<td>4</td>
<td>DBN → SA</td>
<td>129 ± 6</td>
<td>411 ± 20</td>
</tr>
<tr>
<td>5</td>
<td>DBN → SE</td>
<td>128 ± 7</td>
<td>422 ± 18</td>
</tr>
<tr>
<td>6</td>
<td>DBN →</td>
<td>127 ± 6</td>
<td>458 ± 24</td>
</tr>
<tr>
<td>7</td>
<td>→ BHA</td>
<td>132 ± 7</td>
<td>368 ± 18</td>
</tr>
<tr>
<td>8</td>
<td>→ BHT</td>
<td>132 ± 6</td>
<td>346 ± 16</td>
</tr>
<tr>
<td>9</td>
<td>→ Ethoxyquin</td>
<td>132 ± 6</td>
<td>362 ± 10</td>
</tr>
<tr>
<td>10</td>
<td>→ SA</td>
<td>131 ± 6</td>
<td>418 ± 21</td>
</tr>
<tr>
<td>11</td>
<td>→ SE</td>
<td>131 ± 6</td>
<td>430 ± 17</td>
</tr>
</tbody>
</table>

* Mean ± SD.

**Table 2 Lesions of the esophagus and forestomach in rats treated with DBN followed by various antioxidants**

*Numbers in parentheses, percentage of rats showing lesions.

**Table 3 Lesions of the liver in rats treated with DBN followed by antioxidants**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Hyperplastic foci</th>
<th>Hyperplastic nodules</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DBN → BHA</td>
<td>18</td>
<td>18 (100)*</td>
<td>1 (6)</td>
<td>3 (18)</td>
</tr>
<tr>
<td>2</td>
<td>DBN → BHT</td>
<td>21</td>
<td>21 (100)</td>
<td>15 (71)</td>
<td>6 (29)</td>
</tr>
<tr>
<td>3</td>
<td>DBN → Ethoxyquin</td>
<td>21</td>
<td>21 (100)</td>
<td>13 (62)</td>
<td>3 (14)</td>
</tr>
<tr>
<td>4</td>
<td>DBN → SA</td>
<td>19</td>
<td>19 (100)</td>
<td>2 (11)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>5</td>
<td>DBN → SE</td>
<td>21</td>
<td>21 (100)</td>
<td>5 (24)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>6</td>
<td>DBN →</td>
<td>21</td>
<td>21 (100)</td>
<td>8 (38)</td>
<td>5 (24)</td>
</tr>
<tr>
<td>7</td>
<td>→ BHA</td>
<td>19</td>
<td>19 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>→ BHT</td>
<td>20</td>
<td>1 (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>→ Ethoxyquin</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>→ SA</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>→ SE</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of rats showing lesions.
contrast, BHT enhanced esophageal carcinogenesis, but did not affect forestomach carcinogenesis. Thus there are different carcinogenic for the forestomach, the effect of BHA in the consistent with the results of BHA in methylnitrosourea-initiated forestomach carcinogenesis (6). Moreover, since BHA is carcinogenic for the forestomach, the effect of BHA in the methylnitrosourea-initiated forestomach carcinogenesis (6). Moreover, since BHA is carcinogenic for the forestomach, the effect of BHA in the 2-yr carcinogenicity study of rats (13, 14).

Recently Ito et al. (13) reported that p.o. administration of 2% BHA in the diet for 2 yr induced forestomach carcinomas in F344 rats. However, treatment of rats with 2% BHA for 32 wk did not induce carcinomas in the forestomach, although it induced papillomas (1). An enhancement by BHA of DBN-initiated forestomach carcinogenesis in the present study was consistent with the results of BHA in methylnitrosourea-initiated forestomach carcinogenesis (6). Moreover, since BHA is carcinogenic for the forestomach, the effect of BHA in the present study might be summation effect with that of DBN. Significantly increased DNA synthesis in the forestomach epithelium was produced by 2% BHA treatment (15-17). In the present study, BrdUrd incorporation in the forestomach epithelium was significantly increased by BHA treatment. These phenomena indicate that BHA can stimulate cell proliferation, which may be related to its enhancement of the forestomach carcinogenesis. The most interesting result in the present study was that BHT promoted esophageal carcinogenesis but not forestomach carcinogenesis. Although BHT tended to increase DNA synthesis of the esophageal epithelium and did not increase DNA synthesis of the forestomach epithelium, further investigations are required to elucidate the mechanism of this action. In addition, both BHA and BHT enhanced bladder carcinogenesis by DBN. This confirmed the results of Imaida et al. (1, 6). However, these 2 antioxidants did not modify liver carcinogenesis by DBN. Treatment with BHT after initiation in previous experiments has resulted in varying effects on the liver of rats, i.e., an increase, decrease, or no effect on the development of preneoplastic or neoplastic lesions (1, 7, 8, 18). Similar treatment with BHA inhibited the induction of preneoplastic lesions in the liver of rats. In the present study, a modifying effect of BHA or BHT on liver carcinogenesis might be masked because of the large dose of DBN used for initiation or because of the short time involved in the second stage. Moreover, lack of modification of liver carcinogenesis may be due to using concentrations of test chemicals that were high and hence toxic to the liver.

Ethoxyquin also significantly enhanced the induction of esophageal papillomas by DBN. This chemical has been shown to promote kidney and bladder carcinogenesis and inhibit liver carcinogenesis in previous experiments (3, 4). However, in the present study, it did not modify liver or bladder carcinogenesis. SA and SE showed potent promoting activity for bladder carcinogenesis of rats initiated with N-butyl-N-(4-hydroxybutyl)nitrosamine or methylnitrosourea (2, 3, 6). In the present study they did not exert enhancing activities for bladder carcinogenesis. The doses of ethoxyquin, SA, and SE were the same as in the previous experiment (2, 3). We have no explanation for these different results, but speculate that the dose of DBN was too large in the present study and that the duration of the modifying process by ethoxyquin, SA, or SE was not adequate.

Table 4 Induction of bladder lesions in rats treated with DBN followed by various antioxidants

Table 5 Labeling index of esophageal and forestomach epithelium in rats fed 2% BHA or 1% BHT for 2 wk

REFERENCES


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