Effects of Butylated Hydroxyanisole on the Tumorigenicity and Metabolism of N-Nitrosodimethylamine and N-Nitrosopyrrolidine in A/J Mice

Fung-Lung Chung, Minyao Wang, Steven G. Carmella, and Stephen S. Hecht

ABSTRACT

Female A/J mice were maintained on NIH-07 diet or on NIH-07 diet containing butylated hydroxyanisole (BHA, a mixture of 2- and 3-tert-butyl-4-hydroxyanisole), 5 mg/g, for 1 week prior to and during 10 weeks of treatment with N-nitrosodimethylamine (NDMA) or N-nitrosopyrrolidine (N PYR), administered in the drinking water. Twenty weeks after nitrosamine treatment ended, mice were sacrificed and lung adenomas were counted. BHA inhibited NDMA tumorigenesis but enhanced NPYR tumorigenesis. Treatment of A/J mice for three weeks with BHA (5 mg/g) added to semisynthetic diet increased whole lung microsomal α-hydroxylation of NDMA and NPYR, as measured by aldehyde production, and increased lung and hepatic glutathione-S-transferase activities. No evidence was found for formation of S-methylglutathione in incubations with NDMA and hepatic supernatants obtained from BHA treated or control mice. Four hours after gavage of NDMA, levels of 7-methylguanine in the lung DNA of mice treated with BHA were higher than in the lung DNA of control mice, but levels of O6-methylguanine in the two groups were the same. The results of this study indicate that BHA treatment increases the microsomal metabolic α-hydroxylation of both NDMA and NPYR, but has differential effects on their tumorigenic activities.

INTRODUCTION

BHA is widely used as a preservative in food; its consumption in the United States has been estimated at 3 mg/day (1). BHA inhibits carcinogenesis by benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene and certain other chemicals (2). However, limited information is available on inhibition of nitrosamine carcinogenesis by BHA. In an abstract, Wattenberg reported that BHA inhibits lung tumorigenesis induced by N-nitrosodiethylamine in A/J mice (3). Witschi and Doherty (4) recently reported that BHA did not inhibit NDMA tumorigenesis in A/J mice. Ito et al. (5) and Tsuda et al. (6) demonstrated that BHA inhibited the development of liver lesions in rats treated with N-nitroso-N-ethyl-N-hydroxyethylamine and N-nitrosodiethylamine, but was a promoter of bladder cancer in rats treated with N-nitroso-N-butyl-N-4-hydroxybutylamine.

Since nitrosamines of various structural types occur widely in respiratory and occupational environments, are present in trace quantities in beverages and food, and can be formed endogenously in man, it is important to assess the possible modifying effects of BHA on their tumorigenic activities (7, 8). In the present study we have investigated the effects of BHA on NDMA and NPYR tumorigenesis and metabolic activation in A/J mouse lung. NDMA and NPYR were chosen because they occur in the environment and are structurally representative of an acyclic and a cyclic nitrosamine.

MATERIALS AND METHODS

Chemicals. BHA, a mixture of 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole was obtained from Sigma Chemical Co. (St. Louis, MO) and was used without purification. NDMA, NPYR, and iodoacetic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). 2-Methylthioglutathione, glutathione, and 4-fluoro-2,4-dinitrobenzene were procured from Sigma Chemical Co.

Tumorigenesis Experiment. Female A/J mice, 9–10 weeks old, obtained from The Jackson Laboratory, Bar Harbor, ME, were divided into groups of 40 mice each. They were housed 10/cage in solid-bottomed polycarbonate cages with hardwood chip bedding under standard conditions [22 ± 2°C (SD); 50 ± 10% relative humidity; 12-h light, 12-h dark cycle]. They were allowed free access to either NIH-07 diet or NIH-07 diet to which 5 mg (0.028 mmol) BHA/g had been added. After 1 week the appropriate nitrosamine was added to the drinking water of the various groups as summarized in Table 1; concentrations were NDMA (5 mg/liter, 0.068 mmol/liter) and NPYR (40 mg/liter, 0.4 mmol/liter). The drinking water was placed in amber bottles and fresh solutions were provided weekly. The stability of the nitrosamines in the drinking water was assessed as follows. The nitrosamine solutions were extracted five times with CH2CL2. The extracts were dried, concentrated, and analyzed on a combined gas chromatography-thermal energy analyzer (Thermedics, Inc., Woburn, MA) equipped with a 12-ft x 2-mm glass column filled with 10% Carbowax 20 M on Chromosorb WAW. The oven temperature was 160°C and the flow rate was 30 ml argon/min. Each analysis was carried out five times. After 7 days of standing at room temperature in the amber bottles, concentrations of NDMA and NPYR were 95.8 and 98.3%, respectively, of their initial concentrations.

Treatment of the mice with nitrosamines continued for 10 weeks. Water consumption was measured during nitrosamine treatment by weighing the bottles every 3 days. There were no significant differences in the water consumption among the groups (Table 1). At the end of the 10-week period of nitrosamine treatment, all mice were returned to normal drinking water and NIH-07 diet. Twenty weeks later the mice were killed and lung adenomas were counted. Statistical significance was evaluated with the two-sample t test and the χ² test.

Microsomal Metabolism of NDMA and NPYR. Fifty female A/J mice, 7–9 weeks old, were maintained on modified AIN-76 semisynthetic diet (9, 10) for 1 week. Then 25 mice were switched to semisynthetic diet containing 5 mg (0.028 mmol) BHA/g while the other 25 mice were maintained on semisynthetic diet. This protocol continued for 3 weeks. The mice were sacrificed and 5 pools of lung microsomes from 5 mice per pool were prepared from each group, as previously described (11). Liver microsomes were prepared from 4 mice from each group.

Lung microsomes were incubated with cofactors and 0.1 μm NDMA for 30 min or with 0.2 μm NPYR for 60 min, and the mixtures were analyzed for formaldehyde or 4-hydroxybutanal as previously described.
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(11, 12). Each assay used a pool of lung microsomes from 5 mice (0.1–0.3 mg protein for NDMA and 0.5–0.8 mg protein for NPYR). Each assay was performed in duplicate. Liver microsomes (1.5–2.2 mg protein) were incubated with 20 nM NPYR for 60 min.

Analyses for Glutathione, S-Methylglutathione, and Glutathione S-Transferase Activity. Female A/J mice were treated with BHA or control diets exactly as described above. Liver and lung cytosols were analyzed for glutathione content and glutathione-S-transferase activity, with 1-chloro-2,4-dinitrobenzene as substrate, as described previously (13, 14). Analyses for formation of S-methylglutathione during the metabolism of NDMA were carried out using liver 10,000 × g supernatants from female A/J mice maintained on BHA-containing or control diets, as above. Incubation mixtures contained NDMA (20 μM), 10,000 × g supernatant (88 mg), and cofactors as in the formaldehyde assay, except that the concentration of NADPH was increased to 6 μM in order to maintain glutathione in the reduced state. Incubations were carried out at 37°C for 60 min. Control incubations lacked NDMA. Reactions were terminated by addition of 0.5 ml 50% trichloroacetic acid. The mixture was centrifuged for 20 min at 10,000 rpm. The supernatant was extracted 4 times with CHCl₃. The aqueous phase (100-μl aliquot) was mixed with 100 μl ethanol, 50 μl aqueous NaHCO₃ (30 μM), and 35 μl of aqueous ICH₂CO₂H (3 μM). After 1-h reaction time at room temperature, 20 μl of 1-fluoro-2,4-dinitrobenzene (1.5% in ethanol) were added and the mixture was allowed to stand overnight. Aliquots were separated by HPLC on a Perkin-Elmer HS-5 C-18 column with a gradient from 50 to 80% Solvent B in Solvent A in 40 min at 1 ml/min. Solvent A was 24 mM, pH 5 acetate buffer, and Solvent B was methanol. Detection was by UV at 365 nm. The region corresponding to derivatized S-methylglutathione (13–18 min) was collected and analyzed on a carbohydrate analysis column (Waters Chromatography Division, Millipore, Inc., Milford, MA) programmed with a gradient from 0 to 68% Solvent B in Solvent A in 30 min at 1 ml/min. Solvent A was 80% methanol in H₂O and Solvent B was 400 ml of Solvent A mixed with 63 ml glacial acetic acid. 25.6 g ammonium acetate, and 20 ml H₂O. Detection was at 365 nm. The retention time of derivatized S-methylglutathione in this system was 14.5 min.

Analyses for mGua and O₆-mGua in Mouse Lung DNA upon Treatment with NDMA. Fifty-one female A/J mice were maintained on semisynthetic diet for 1 week. Twenty-one of these mice were maintained on this diet for an additional 3 weeks. Thirty mice were given semisynthetic diet containing 5 mg BHA/g for 3 weeks. On the last day of treatment the 21 control mice and 21 of the BHA treated mice were administered 300 μg (4 μmol) of NDMA/mouse by gavage in 0.2 ml saline. Nine BHA treated mice were treated with saline only. Four h later all mice were killed by cervical dislocation. Lungs from pools of 3 mice each were excised for DNA isolation. DNA was isolated using the modified Marmur method (15) and levels of O₆-mGua, mGua, and guanine were determined by HPLC with fluorescence detection as previously described (16).

RESULTS

The results of the tumorigenesis experiment are summarized in Table 1. Based on the number of tumors per mouse, BHA significantly inhibited induction of lung adenomas by NDMA. In contrast, BHA significantly increased both the percentage of tumor bearing mice and the number of lung tumors per mouse in the animals treated with NPYR. A small but statistically significant increase in the number of lung adenomas per mouse was observed in the group treated with BHA only, compared to the control group.

Since opposing effects of BHA were observed on the tumorigenicity of NDMA and NPYR, we investigated its effects on their metabolic activation. The initial activation step for NDMA and NPYR is thought to be an α-hydroxylation producing formaldehyde from NDMA and 4-hydroxybutanal from NPYR. As shown in Table 2, BHA pretreatment increased lung microsomal aldehyde production from both nitrosamines. Since the rates of lung microsomal NPYR α-hydroxylation were low, we also carried out the assay with liver microsomes; an increase in production of 4-hydroxybutanal was observed.

It is well established that BHA can induce glutathione-S-transferases in mice (17, 18). These activities had not been previously measured in A/J mice. The effects of dietary BHA on glutathione-S-transferase activity and glutathione content in A/J mouse liver and lung are summarized in Table 3. Glutathione-S-transferase activity toward 1-chloro-2,4-dinitrobenzene was significantly increased in the BHA treated animals; glutathione content was unchanged.

Since glutathione-S-transferase activity was increased by BHA, it seemed possible that methylating intermediates produced in the metabolic activation of NDMA might be scavenged more effectively in BHA treated mice than in controls. To determine whether S-methylglutathione was formed during the metabolism of NDMA, and if its levels were influenced by BHA treatment, we developed a HPLC method for detection of S-methylglutathione in incubation mixtures of NDMA, cofactors, and mouse hepatic 10,000 × g supernatant. The method is similar to that described for glutathione (19). A peak corresponding in

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### Table 1

**Effects of BHA on induction of lung tumors in A/J mice by NDMA and NPYR**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Daily water consumption (ml/mouse)</th>
<th>Mean wet g</th>
<th>% of mice with lung tumors</th>
<th>Lung tumors/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>11 wk&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29 wk&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDMA</td>
<td>40</td>
<td>3.3 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.2</td>
<td>100</td>
<td>18.6 ± 13.4</td>
</tr>
<tr>
<td>NPYR</td>
<td>40</td>
<td>3.2 ± 1.3</td>
<td>22.0</td>
<td>93</td>
<td>4.6 ± 3.5</td>
</tr>
<tr>
<td>NPYR + BHA</td>
<td>40</td>
<td>3.6 ± 0.2</td>
<td>22.7</td>
<td>87</td>
<td>1.85 ± 1.25</td>
</tr>
<tr>
<td>BHA</td>
<td>38</td>
<td>3.4 ± 0.5</td>
<td>23.0</td>
<td>87</td>
<td>1.35 ± 1.11</td>
</tr>
<tr>
<td>Control</td>
<td>40</td>
<td>3.2 ± 1.1</td>
<td>23.2</td>
<td>29</td>
<td>0.42 ± 0.92</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number at autopsy.
<sup>b</sup> Mean ± SD.
<sup>c</sup> Weeks of treatment.
<sup>d</sup> Significant differences: NDMA versus control, P < 0.01; NPYR versus NPYR + BHA, P < 0.01.
<sup*e</sup> Significant differences: NDMA versus NDMA + BHA, P < 0.001; NPYR versus NPYR + BHA, P < 0.001; NDMA versus control, P < 0.001; NPYR versus control, P < 0.05; BHA versus control, P < 0.05.

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Table 2

Effects of BHA treatment on the microsomal α-hydroxylation of NDMA and NPYR in A/J mouse tissues

Female A/J mice were maintained for 3 weeks on control semisynthetic diet or on semisynthetic diet containing 5 mg BHA/g. Lung or liver microsomes were prepared and assayed for α-hydroxylation of NDMA or NPYR as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NDMA (nmol formaldehyde/min/mg protein)</th>
<th>NPYR (nmol 4-hydroxybutanal/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.32 ± 0.06</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>BHA</td>
<td>0.53 ± 0.14</td>
<td>0.39 ± 0.06</td>
</tr>
</tbody>
</table>

*Mean ± SD, n = 4–5 microsomal preparations. Each microsomal preparation represents a pool of lung microsomes from 5 mice and each assay was performed in duplicate.

**Mean ± SD, n = 4. Each microsomal preparation was obtained from a single mouse and each assay was performed in duplicate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver</th>
<th>Lung</th>
<th>Gavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>743 ± 96</td>
<td>257 ± 80</td>
<td>1.7 ± 2.2</td>
</tr>
<tr>
<td>BHA</td>
<td>6670 ± 1370</td>
<td>498 ± 102</td>
<td>3.7 ± 2.2</td>
</tr>
</tbody>
</table>

*Mean ± SD, n = 6 except liver glutathione (n = 4).

**Significantly greater than control, P < 0.001.

Table 3

Effects of BHA treatment on glutathione-S-transferase activity and glutathione content in A/J mouse liver and lung

Female A/J mice were maintained for 3 weeks on control semisynthetic diet or on semisynthetic diet containing 5 mg BHA/g. Liver or lung cytosols were analyzed for glutathione-S-transferase activity with 1-chloro-2,4-dinitrobenzene as substrate or for glutathione content as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glutathione-S-transferase (nmol/min/mg protein)</th>
<th>Glutathione (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>84.7 ± 12.0</td>
<td>2.8 ± 1.0</td>
</tr>
<tr>
<td>BHA</td>
<td>94.6 ± 10.7</td>
<td>3.8 ± 1.0</td>
</tr>
</tbody>
</table>

*Mean ± SD, n = 6 except liver glutathione (n = 4).

**Significantly greater than control, P < 0.001.

Table 4

Effects of BHA treatment on levels of O6-mGua and O6-mGua in A/J mouse lung, 4 h after treatment with NDMA

Female A/J mice were maintained for 3 weeks on either control semisynthetic diet or semisynthetic diet containing 5 mg BHA/g. They were then treated with 300 μg of NDMA in 0.9% saline by gavage. DNA was isolated from lung and analyzed for mGua and O-mGua as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mGua (μmol/mol guanine)</th>
<th>O-mGua (μmol/mol guanine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>241 ± 79</td>
<td>31 ± 10</td>
</tr>
<tr>
<td>BHA</td>
<td>353 ± 94</td>
<td>36 ± 5</td>
</tr>
</tbody>
</table>

*Mean ± SD from 7 pools of lung DNA. Each pool consisted of lungs from 3 mice.

**Significantly greater than control, P < 0.04.

retention time to derivatized S-methylglutathione was observed in incubation mixtures containing NDMA, but a peak of comparable size was also observed in control incubations lacking NDMA. S-Methylglutathione added to incubation mixtures of cofactors and mouse hepatitis 10,000 × g supernatant was recovered (recovery, >50%). Considering our limits of detection (0.1 ppm), less than 0.001–0.004% of the NDMA present in the incubation mixtures was converted to S-methylglutathione in hepatic supernatants from either control or BHA treated mice. This corresponds to less than 3% of the NDMA metabolized under these conditions.

Table 4 summarizes the effects of BHA on levels of mGua and O6-mGua in lung DNA, 4 h after treatment of A/J mice with NDMA. Levels of mGua were significantly higher in the BHA treated mice than in controls; no difference was observed in O6-mGua levels. In the same experiment, 9 BHA treated mice were not given NDMA; mGua (8 ± 7 μmol/mol guanine) but not O6-mGua was detected in lung DNA. Thus BHA apparently induced a low level of aberrant methylation.

DISCUSSION

The results of the bioassay show that BHA significantly inhibited mouse lung tumorigenesis induced by NDMA when judged by the number of tumors per animal. It is likely that at lower doses of NDMA the percentage of tumor bearing animals would also have decreased. The results of a recent study by Witschi and Doherty (4) are different from those reported here. In their study, BHA given after a single dose of NDMA had no effect on lung tumor incidence. However the results were difficult to evaluate because the single dose (7 mg/kg) of NDMA, combined with BHA treatment, caused the death of 17 of the 25 NDMA treated mice. In a second experiment, BHA given for 2 weeks prior to a single dose (7 mg/kg) of NDMA had no effect on tumor multiplicity; in that experiment survival was excellent (4).

In order to gain insight into the contrasting effects of BHA on NDMA and NPYR tumorigenesis, we carried out metabolic studies in BHA treated and control mice. In A/J mouse lung, BHA increased the microsomal α-hydroxylation of both NDMA and NPYR, as measured by aldehyde production. Four h after treatment with NDMA, levels of mGua were higher in lung DNA of BHA treated mice than in control mice. This observation is consistent with the observed increase in microsomal α-hydroxylation of NDMA, which produces 1 mol of methylating agent per mol of formaldehyde, and with the lack of any apparent scavenging of methylating agents by glutathione. Several lines of evidence support the hypothesis that α-hydroxylation is the initial metabolic activation step for both NDMA and NPYR (7, 8, 11, 20, 21). Therefore, based on these observations alone, increased tumorigenicity of NDMA and NPYR might have been expected in the BHA treated mice compared to the control mice. However, BHA decreased the tumorigenicity of NDMA. The effects of BHA on DNA methylation by NDMA might partially explain this apparent discrepancy. Levels of O6-mGua were not significantly different in the lung DNA of the BHA treated and control groups. This suggests that BHA treatment may have increased the capacity of A/J mouse lung to repair O6-mGua, possibly resulting in its lower persistence. Further studies are required to elucidate the mechanism of the protective effect of BHA on NDMA tumorigenesis. It will be important to carry out these studies in type II alveolar epithelial cells, which are the target cells for adenoma formation in the A/J mouse (22).

Presently available methods are not sensitive enough to measure NPYR-DNA adduct formation in A/J mouse lung. Based on the results of the in vitro α-hydroxylation assay, it would be expected that higher initial levels of NPYR-DNA adducts would be present in BHA treated than in control mice. The results of the tumorigenesis study suggest that NPYR-DNA adducts are not readily repaired or that BHA has no effect on their repair.

In summary, this study has demonstrated that the effects of BHA on nitrosamine tumorigenesis and metabolism in A/J mouse lung depend on the structure of the nitrosamine. Humans are exposed to mixtures of nitrosamines. The way in which dietary BHA may modify the potential carcinogenicity of such mixtures will depend on the concentrations of particular nitrosamines in these mixtures and on the specific effects of BHA on each. Further studies are required to evaluate the overall potential of
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BHA as a preventive or enhancing agent in environmental nitrosamine carcinogenesis.

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REFERENCES