Short Time Interval Effects of Butylated Hydroxyanisole on the Metabolism of Benzo(a)pyrene

Luke K. T. Lam, Alan V. Fladmo, J. Bradley Hochalter, and Lee W. Wattenberg

Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT

Within 4 hr after administration of butylated hydroxyanisole (BHA) by p.o. intubation, marked changes in the microsomal metabolism of benzo(a)pyrene (BP) occur. Liver microsomes isolated from female A/HeJ mice under these conditions show a depression of BP metabolism by more than 16%. The effects on individual metabolites as determined by high-pressure liquid chromatography differ. Relative increases in 3-hydroxybenzo(a)pyrene and in the dione regions were observed. In contrast, benzo(a)pyrene 4,5-oxide formation was decreased greater than 30%. trans-4,5-Dihydroxy-4,5-dihydrobenzo(a)-pyrene underwent a similar decrease while the other two diols, trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene and trans-9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene showed no significant alteration. The most profound decrease was in the concentration of metabolites in the very polar region of the chromatogram. The retention times of some of these peaks coincide with those of the hydrolysis products of BP diol-epoxides, i.e., tetrols and triols. A reduction in these highly polar metabolites is a good indication that the formation of diol-epoxides was inhibited by BHA. Thus, BHA alters microsomal metabolism by diminishing activation reactions leading to the formation of ultimate carcinogenic metabolites and also enhances formation of metabolites of detoxification. Along with the change in BP metabolite pattern, BHA given in vivo induces a different response of microsomes to subsequent in vitro addition of BHA. The capacity of microsomes to undergo rapid changes that overall diminish formation of carcinogenic metabolites could constitute an important protective mechanism.

INTRODUCTION

BHA is a phenolic antioxidant that inhibits the neoplastic effects of many chemical carcinogens including BP (14). When added to the diet, BHA suppresses the formation of pulmonary adenomas and neoplasms of the forestomach in mice (12, 13). Studies of the mechanism of inhibition of neoplasia under these conditions have shown that BHA alters the metabolism of BP in a manner which would favor detoxification. Thus, incubation of BP with liver microsomes isolated from mice fed BHA results in less binding of BP metabolites to added DNA than occurs with microsomes from control mice (11). The BP metabolite pattern found on incubating BP with microsomes from mice fed BHA is altered (8). There is a decrease in epoxidation of BP, which is an activation process, as well as an increase in the formation of 3-HOBP, a major metabolite of detoxification. Other changes involving the cytochrome P-450 system are observed (11). The amount of cytochrome P-450 per mg of liver microsomal protein is increased by feeding BHA. There is a change in the ethyl isocyanide difference spectra and an increase in sensitivity to inhibition by β-naphthoflavone. Further work has demonstrated that mice consuming a diet containing BHA have a marked increase in the activities of 2 conjugating enzymes, glutathione S-transferase (1, 2) and UDP-glucuronotransferase (3), as well as epoxide hydratase (2-4, 7). In addition, there is an increase in tissue glutathione levels (2).

In subsequent work, experiments were performed to determine how the time relationship between administration of BHA and the carcinogen would alter the protective effect of the antioxidant. For this purpose, single administrations of BHA by p.o. intubation were used (10). Under these conditions, it was found that BHA inhibited pulmonary adenoma formation when given 4 hr prior to BP, the shortest time interval studied. The suppression of neoplasia was similar in magnitude to that of the feeding experiments. Incubation of BP with liver microsomes from mice that had received BHA by p.o. intubation 4 hr and even 2 hr before the animals were killed resulted in a decrease in binding of BP metabolites to added DNA. Again, the magnitude of the decrease was the same as in the experiments in which BHA was fed in the diet. Thus, it became apparent that BHA produces inhibitory effects shortly after administration. The present investigation is an effort at obtaining data which would be useful in elucidating the mechanism(s) by which BHA produces these short time interval inhibitory effects. Studies of the microsomal metabolism of BP have been carried out. In addition, the response of microsomes to the in vitro addition of BHA has been determined.

MATERIALS AND METHODS

Chemicals. BHA (>99% purity; Sigma Chemical Co., St. Louis, Mo.) contains a mixture of 2 isomers was used without further purification. BP was obtained from Aldrich Chemical Co., Milwaukee, Wis. NADPH was obtained from P-L Biochemicals, Milwaukee, Wis. Glass-distilled solvents for HPLC were obtained from Burdick & Jackson Laboratories, Inc., Muskegon, Mich.

Radiochemicals. [3H]BP (25 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) was diluted with unlabeled BP to approximately 65 mCi/mmol. The diluted sample was purified by passing through a silica gel column and elution with benzene. The solvent was removed by freeze-drying, and the solid
sample was stored at −65° under nitrogen. The purity was determined by HPLC to be greater than 99.8%.

**Incubations.** Forty 10- to 12-week-old A/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine) were divided equally into 2 groups in each experiment. In the short time interval experiments, one group of mice was given 7.5 mg of BHA in 0.1 ml of cottonseed oil by p.o. intubation 4 hr before the mice were killed. The second group, i.e., the controls, was given 0.1 ml of cottonseed oil. In one set of studies, mice were fed BHA in the diet. In this instance, the BHA group was fed a diet containing 5 mg of BHA per g of ground Purina Rat Chow (Ralston-Purina Co., St. Louis, Mo.), and the control group was fed with ground Purina Rat Chow without any additions. In all experiments, the liver microsomes were prepared as previously described (11). To a reaction vial containing 4 ml of 0.05 M phosphate buffer were added 80 μg of [3H]BP (=65 mCi/mmole) with or without the addition of BHA in 100 μl of acetone, 10 mg of protein equivalent of liver microsomes in 1 ml of 1.15% KCl, and 4 mg of NADPH in 1 ml of phosphate buffer. The reaction mixture was incubated at 37° for 20 min. The reaction was terminated by the addition of 3 ml of acetone and 9 ml of ethyl acetate. The reaction vial was vigorously shaken, and the contents were transferred into a centrifuge tube. The organic layer was removed after centrifugation for 10 min at 2000 rpm. The aqueous layer was further extracted with 5 ml of ethyl acetate. The organic layers were combined, and the solvent was removed by freeze-drying. The crude extract was stored at −65° under nitrogen until HPLC analysis.

**HPLC Analysis.** The analysis of microsomal incubation extracts was similar to that previously described (8). The incubation extract was dissolved in 0.1 ml of tetrahydrofuran, and an aliquot of 20 μl was injected into a Waters Associates Model 202 high-pressure liquid chromatograph equipped with a gradient solvent programmer. Two C18Bondapak columns (Waters Associates, Milford, Mass.) in series were used with reverse-phase gradient solvent elution of 55 to 75% methanol at ambient temperature over a period of 1.5 hr. One-ml fractions were collected, and the radioactivity of each fraction was determined by liquid scintillation counting. Data obtained from the liquid scintillation spectrophotometer were converted to dpm with the necessary quench correction by a CDC Cyber 172 computer. The dpm versus fraction number graphs were plotted by means of a Varian Statos 42 electrostatic plotter. Computer programs were written to calculate the percentage of individual metabolites as well as the percentage of total metabolism and to obtain the BHA:control ratio of these percentages. The metabolites were identified by comparing their HPLC retention times with those of the authentic samples of BP derivatives, supplied by the Chemical Repository, National Cancer Institute.

**RESULTS**

The total microsomal metabolism of BP from mice given BHA by p.o. intubation 4 hr prior to sacrifice was 6 to 8%. Microsomes from control mice gave a slightly higher total metabolism of BP, i.e., 8 to 14%. In 3 separate experiments, the reduction of total BP metabolism due to BHA treatment was greater than 16%. The highest observed was 42% (Table 1).

The percentages of individual BP metabolites determined by their radioactivity distribution from HPLC analysis are given in Table 2. There is a slight increase in the percentage of 3-HOBP and 9-HOBP in the BHA group. Other increases observed were in the dione peaks and an unknown peak which eluted between the 6,12-dione of BP and the 9-HOBP peaks. The remaining metabolites showed either a significant decrease or no change as a result of BHA treatment. BP 4,5-oxide formation was decreased greater than 30%. The corresponding BP 4,5-diol underwent a similar decrease while the other 2 diols showed no significant alteration. The polar region (Table 2; Peaks 2, 3, and 4) which contains metabolites of diols and oxides of BP was diminished by 40%.

Studies of the effects of in vitro addition of BHA into the microsomal incubation mixture were carried out to compare with alterations brought about by administration of BHA in vivo. The in vitro addition of BHA into the microsomal incubation mixture depressed the overall metabolism of BP. The profile of the metabolite pattern did not change appreciably, which is in marked contrast to the findings following in vivo administration of BHA (Chart 1). Differences in the magnitude of inhibition occurred when BHA was added in vitro to microsomes from control mice as compared to those which had received BHA in vivo. With microsomes from control mice, addition of BHA at a concentration of 15 μg/incubation resulted in a 44% decrease in the total metabolism of BP. A smaller inhibition, 32%, was obtained when microsomes from mice given BHA by p.o. intubation were used. At higher concentration of BHA, i.e., 40 μg/incubation, the inhibitory effects were more pronounced. The total BP metabolism by control microsomes with in vitro addition of BHA was only 20% of that without BHA being present. The total BP metabolism by microsomes from the mice given BHA was 45% that of the incubation without added BHA (Table 3).

A plot of the percentage of BP metabolism versus in vitro BHA concentration is given in Chart 2. The in vitro BHA concentration varied from 0 to 15 μg/incubation in the first experiment and from 0 to 40 μg/incubation in the second experiment. At these concentration levels, the percentage of BP metabolism as a function of in vitro BHA addition is expected to be linear (15). A least-squares method of analysis was used so that the percentage of BP metabolism versus the concentration of BHA is plotted as straight lines. The slopes of the plots from the data obtained with control microsomes are twice those of the plots with data from BHA microsomes, indicating an altered enzyme response as a result of treatment with BHA in vivo, in this instance by p.o. intubation.
Table 2
Radioactivity distribution of BP metabolites expressed in percentage of the total metabolites

<table>
<thead>
<tr>
<th>Material administered</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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</thead>
<tbody>
<tr>
<td>BHA</td>
<td>1.14</td>
<td>0.91</td>
<td>1.34</td>
<td>2.68</td>
<td>0.50</td>
<td>1.14</td>
<td>0.91</td>
<td>1.34</td>
<td>2.68</td>
<td>0.50</td>
</tr>
<tr>
<td>CON</td>
<td>1.10</td>
<td>1.42</td>
<td>1.29</td>
<td>2.25</td>
<td>0.57</td>
<td>1.10</td>
<td>1.42</td>
<td>1.29</td>
<td>2.25</td>
<td>0.57</td>
</tr>
<tr>
<td>BHA:CON</td>
<td>0.54</td>
<td>0.44</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.54</td>
<td>0.44</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
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Table 3
Effect of in vitro addition of BHA on the total BP metabolism by BHA and control microsomes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>In vitro BHA concentration (µg)</th>
<th>% of BP metabolism</th>
<th>% of inhibition</th>
<th>% of BP metabolism</th>
<th>% of inhibition</th>
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<td>1</td>
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<td>5.92</td>
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<td>10.20</td>
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<td></td>
<td>3.5</td>
<td>5.67</td>
<td>4</td>
<td>8.33</td>
<td>18</td>
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<tr>
<td></td>
<td>7.0</td>
<td>6.44</td>
<td>-7</td>
<td>8.26</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>4.04</td>
<td>32</td>
<td>5.70</td>
<td>44</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>7.71</td>
<td>0</td>
<td>11.74</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>7.36</td>
<td>5</td>
<td>12.18</td>
<td>-3</td>
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<tr>
<td></td>
<td>40.0</td>
<td>3.48</td>
<td>55</td>
<td>2.34</td>
<td>80</td>
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Comparative experiments have been carried out using microsomes from mice fed BHA in the diet. Results similar to those in which the BHA was administered by p.o. intubation were obtained (Table 4). Again, there was a reduction of BP metab-
The mechanism by which BHA brings about the short time interval alteration in the microsomal metabolism of BP remains to be established. Some information bearing on this process which can be determined directly is BP 4,5-oxide, and there was a marked reduction in the formation of this metabolite. The corresponding diol, BP 4,5-diol, likewise was reduced. The formation of the other 2 diols, BP 7,8-diol and BP 9,10-diol, did not show significant deviation from that of control levels. The data obtained indicate that the decrease in BP 4,5-oxide is due to a reduction in the primary metabolism of BP, i.e., the epoxidation of the carcinogen. Cha et al. (4) reported an enhancement of liver microsome epoxide hydratase activity in mice fed BHA in the diet for 3 days. Single-dose short-term effects of BHA on this enzyme system have not been reported. An increase in epoxide hydratase activity would explain the decrease of BP 4,5-oxide if BP 4,5-diol showed a corresponding increase. The fact that BP 4,5-diol formation was also reduced supports our previous conclusion that BHA treatment reduces the primary metabolism of BP (8). The formation of BP 7,8-diol and BP 9,10-diol did not show significant deviation from that of control levels and further indicates the lack of differential epoxide hydratase action on the BP oxides in microsomes from control versus BHA-treated mice. Administration of BHA by p.o. intubation 4 hr prior to sacrifice resulted in an increase in the formation of 3-HOBP, 9-HOBP, and the region containing quinones. The HPLC retention times of 6-HOBP and 9-HOBP are degenerate under the present conditions of analysis (8). The higher percentages of labeled material in the 9-HOBP region were probably due to the presence of more unoxidized 6-HOBP in samples from the BHA-treated mice. The quinones are the further oxidation products of 6-HOBP. The relative concentrations of these quinones obtained in this study are consistent with the findings reported by Lesko et al. (9) that 6-HOBP oxidation leads to the 1,6-, 3,6-, and 6,12-diones of BP in an approximate 3:3:1 ratio, respectively. An important finding in the present study is that the administration of BHA by p.o. intubation results in a marked decrease in concentration of metabolites in the very polar region of the chromatogram. According to the HPLC studies of Yang and Gelboin (16) and Yang et al. (17), this region contains some of the hydrolysis products of the BP diol-epoxides, i.e., tetrols and triols of BP. A reduction of 40% was observed. This inhibition was also detected in the BHA-feeding experiments. A reduction of this polar region is a good indication that the further metabolism of the diols and oxides of BP was inhibited by BHA treatment. Since the diol-epoxides of BP are ultimate carcinogenic metabolites of BP (5, 6), this reduction would indicate a diminished carcinogenic impact. The results of the present investigation show that administration of BHA produces an altered microsomal metabolism of BP within 4 hr after administration of the phenol. The nature of this metabolic alteration is such as to reduce activation of the carcinogen to ultimate carcinogenic species and to enhance detoxification. The reduced primary epoxidation and the finding of a marked decrease in tetrols and triols are evidence for decreased activation of BP to ultimate carcinogenic metabolites. The increase in 3-HOBP is an enhanced formation of an important metabolite of detoxification.

**DISCUSSION**

The administration of BHA by p.o. intubation 4 hr prior to sacrifice resulted in pronounced changes in the metabolism of BP by liver microsomes. The total metabolism of BP was reduced. The average percentage of reduction from 3 experiments was 30%. In 2 separate experiments, the reduction ranged from 16 to 42%. The percentage of reduction determined by HPLC in this study was slightly greater in magnitude than that of the previous report in which BP metabolism was measured by the aryl hydrocarbon (BP) hydroxylase assay method (10)

While the total metabolism of BP was decreased by BHA administration, the effects on individual metabolites differed. Some were reduced, others increased, and still others did not show significant changes from control levels. The only epoxide

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**Table 4**

<table>
<thead>
<tr>
<th>In vitro BHA concentration (μg)</th>
<th>% of BP metabolism</th>
<th>% of inhibition</th>
<th>% of BP metabolism</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.10</td>
<td>0</td>
<td>9.01</td>
<td>0</td>
</tr>
<tr>
<td>3.5</td>
<td>7.46</td>
<td>-5</td>
<td>9.28</td>
<td>-3</td>
</tr>
<tr>
<td>7.0</td>
<td>6.54</td>
<td>8</td>
<td>7.2</td>
<td>20</td>
</tr>
<tr>
<td>15.0</td>
<td>6.26</td>
<td>12</td>
<td>5.84</td>
<td>35</td>
</tr>
<tr>
<td>35</td>
<td>3.95</td>
<td>44</td>
<td>3.40</td>
<td>62</td>
</tr>
</tbody>
</table>

* BHA and control microsomes were prepared from mice fed 5 mg BHA per g diet or ground rat chow, respectively, for 2 weeks.
* Total incubation volume, 6 ml.
* The values for percentage of BP metabolism are the averages of 2 experiments. Each in vitro BHA concentration contains 2 or more analytical samples.

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**Chart 3.** Least-squares analysis of percentage of BP metabolism versus in vitro addition of BHA to microsomal incubation. —— experiments with microsomes from control mice (A, slope = -0.17); —— experiments with microsomes from mice fed BHA (B, slope = -0.096).
has been obtained. The effects of *in vitro* addition of BHA on the microsomal metabolism of BP differ in microsomes isolated from control mice as compared to those from animals that had received BHA by p.o. intubation. The BP-metabolizing system of microsomes from control mice is more sensitive to inhibition by BHA added *in vitro* (Charts 2 and 3). The slope of the curve from the controls is twice that found with incubation of microsomes from the BHA-treated mice. Residual BHA in the microsomes from *in vivo* administration should not change the slope of the response curve of BP metabolism by BHA microsomes when compared to that of the control microsomes. The result is more consistent with the explanation that there is a rapid *in vivo* alteration of the metabolizing enzymes caused by BHA. This alteration was found in long-term BHA-feeding experiments as well as in the short time interval studies. In the case of long-term feeding, a number of other detoxification enzymes (such as glutathione S-transferase, epoxide hydratase, and UDP-glucurononyltransferase) are induced and have the potential of protecting the animal (1–4, 7). In the case of short-term p.o. intubation, a rapid alteration of microsomal metabolism of BP occurs before induction of some of these detoxification systems takes place and may be responsible for the protective effect of BHA, at least under these conditions.

For an organism to be protected from the noxious effects of repeated exposures to toxic chemicals, an enhancement of detoxification capacity may be required in some instances. A rapid increase in detoxification capability could be important. Increased activity of detoxification enzymes resulting from induction of formation of new enzymes is an effective mechanism for enhancing protection but is relatively slow. BHA can produce protection of both types which may account for its ability to inhibit the noxious effects of such a wide variety of chemicals.

REFERENCES

Short Time Interval Effects of Butylated Hydroxyanisole on the Metabolism of Benzo(\textit{a})pyrene


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