SHORT COMMUNICATION

The plasticizer benzyl butyl phthalate (BBP) inhibits 7,12-dimethylbenz[a]anthracene (DMBA)-induced rat mammary DNA adduct formation and tumorigenesis

K. Singleary, C. MacDonald and M. Wallig

1Department of Food Science and Human Nutrition and 2Department of Veterinary Pathobiology, University of Illinois, Urbana, IL 61801, USA

Although the risk for cancer is multifactorial, a substantial portion of cancer incidence rates is related to environmental factors, including diet and environmental chemicals. The magnitude of the contribution to cancer of the breast from exposure to environmental chemicals remains unclear. The phthalate ester plasticizers are abundantly-produced industrial chemicals that have become widely-dispersed environmental pollutants. The present studies were conducted to determine the effect of the phthalate ester, benzyl butyl phthalate (BBP) on mammary gland carcinogenesis induced in the female rat by the polycyclic aromatic hydrocarbon (PAH) 7,12-dimethylbenz[a]anthracene (DMBA). Exposure to BBP (i.p. injection) at 100 and 500 mg/kg doses for 5 days resulted in a significant 72 and 92% inhibition, respectively, in the in vivo formation of mammary DMBA-DNA adducts, compared to controls. Treatment with BBP (i.g. intubation) for 7 days resulted in a significant (48%) inhibition in mammary DMBA-DNA adduct formation only for those animals receiving the 500 mg/kg dose, compared to controls. Administration of BBP (i.g.) at 500 mg/kg for 7 days also was associated with a significant 8.5-fold increase in the liver activity of 7-ethoxyresorufin-O-deethylase. No change in liver glutathione-S-transferase activity was observed for animals treated with both BBP (i.g.) doses. Treatment with BBP (i.g.) at 250 and 500 mg/kg doses for 7 days prior to DMBA administration resulted in a significant 37% decrease in mammary tumor incidence for both doses, compared to controls. The number of mammary adenocarcinomas per rat was significantly inhibited by 60 and 70% for rats exposed to BBP at the 250 and 500 mg/kg doses, respectively, compared to controls. Therefore, the present studies indicate that BBP acts as a blocking agent toward DMBA-induced rat mammary DNA adduct formation and mammary carcinogenesis. This effect partly may be due to increased metabolism of BBP in the liver. These results underscore the need to further examine the effect of BBP and other phthalates on the various stages of mammary carcinogenesis, as well as on the metabolism of mammary carcinogens.

Among American women, cancer of the breast is a leading cause of cancer-related death. Although the risk for cancer is multifactorial, a substantial portion of cancer incidence rates is related to environmental factors, including diet and environ-

mental chemicals (1,2). The magnitude of the contribution to cancer of the breast from exposure to environmental chemicals remains unclear. Therefore, the identification of environmental agents that can initiate, promote or otherwise influence breast cancer development is important in order to better determine the magnitude of risk due to these pollutants. For example, considerable attention has focused recently on the role of DDT and other related environmental contaminants, which, acting as xenobiotic estrogens (xenoestrogens), are hypothesized to increase cancer risk (3,4). In contrast to pesticides, the phthalate esters are one of the most abundant of industrially-produced chemicals, that have become widely-dispersed environmental pollutants. This group of chemicals is used to improve flexibility of plastic products. It has been reported that humans could be exposed to them via contaminated soil and water, as well as food (5,6). Due to the widespread use of plasticizers in wrappers, food has become a major route of exposure. In the United States, it has been estimated that the levels of phthalates in foods can vary between 50–500 µg/kg. In Great Britain some confectionary products and snacks packaged with plastic wrap were reported to contain phthalate levels of 14 mg/kg (7,8). During the past two decades, these compounds have been evaluated for their potential to adversely impact on human health. Although phthalates appear not to be genotoxic, there has been evidence that at high concentrations they can lead to testicular toxicity and embryolethality in rodents (9–11). It has been suggested that the reproductive toxicity observed in female and male rats exposed to certain phthalates partly may be due to an estrogenic effect (12,13). There is limited and inconsistent information on the role of these phthalates in modifying the process of carcinogenesis. Certain phthalates have been reported to enhance liver and skin carcinogenesis in rodents, and to act as weak estrogens toward human breast cancer cells (12,14–17). Yet, others have observed that exposure to a phthalate after initiation with a hepatocarcinogen leads to an inhibition of preneoplastic lesions and hepatocellular carcinomas (18,19).

In light of the current concern about the role of environmental contaminants in enhancing breast cancer risk, the present studies were conducted to determine the effect of a phthalate ester, benzyl butyl phthalate (BBP*, Figure 1), on chemically-induced rat mammary carcinogenesis. Three experiments were conducted to examine the capacity of one group of contaminants, represented by BBP, to influence mammary gland carcinogenesis induced in the female rat by another group of contaminants, polycyclic aromatic hydrocarbons (PAH), using 7,12-dimethylbenz[a]anthracene (DMBA) as a model. In the first two experiments, animals were treated with BBP at doses of 100 or 500 mg/kg body wt via either intraperitoneal (i.p.) injection (experiment 1) or intragastric (i.g.) intubation (experiment 2), in order to determine its capacity to affect the in vivo formation of DMBA-DNA adducts in the rat mammary gland. The BBP doses were selected to be nontoxic and not to enhance spontaneous mammary tumorigenesis. In this regard,
others have reported that i.p. administration of BBP to mice at doses up to 800 mg/kg for 24 weeks resulted in no significant toxic effects (5). Dietary levels of BBP up to 12,000 ppm for 105 weeks have been demonstrated not to cause spontaneous breast tumor development in female mice and rats (14). For experiment 1, BBP was administered i.p. to female Sprague–Dawley rats ($\bar{x} = 144$ g) daily ($n = 5$/group) for 5 days prior to intubation with DMBA (i.g., 31 mg/kg in corn oil) at 50 days of age. For experiment 2, BBP was administered i.g. to female rats ($\bar{x} = 144$ g; $n = 6$/group) daily for 7 days prior to dosing with DMBA (i.g., 31 mg/kg in corn oil) at 50 days of age. For both experiments BBP was given in corn oil and controls received vehicle only. At 24 h after DMBA dosing in both adduct studies, mammary epithelial cell aggregate DNA was isolated and DMBA-DNA adducts quantitated by the $^{32}$P-post-labeling procedure (20). Also, livers from the animals intubated with BBP were removed, and cytosolic and microsomal fractions prepared for use in subsequent analyses of glutathione-S-transferase (GST) activity (21) and ethoxyresorufin-O-deethylase (EROD) activity (22), respectively.

In experiment 3, the effect of BBP exposure on the initiation stage of DMBA-induced mammary carcinogenesis was evaluated. Female rats ($\bar{x} = 141$ g; $n = 27$/group) were administered BBP at doses of 250 and 500 mg/kg (i.g. in corn oil) daily for 7 days prior to dosing with DMBA (i.g., 31 mg/kg in corn oil) at 50 days of age. A higher dose of 250 mg/kg, instead of 100 mg/kg, was chosen as an intermediate dose for this experiment, because of the lack of effect of the 100 mg/kg dose in inhibiting adduct formation in experiment 2. Controls were intubated with corn oil vehicle prior to DMBA dosing. Animals were weighed weekly and, beginning 5 weeks post-DMBA, were palpated weekly to determine mammary tumor formation. At the termination of the experiment, tumors were removed and classified histopathologically (23). For all three experiments animals were fed a control, semipurified diet, which contained the following ingredients (% by wt) as recommended by the American Society for Nutritional Sciences (AIN76A): vitamin-free casein (20), dl-methionine (0.3), sucrose (25), cellulose (5), AIN-76 mineral mix (3.5), cornstarch (37), choline dihydrogen citrate (0.2) and corn oil (8). In general, animals were acclimated to this diet for at least 1 week prior to the beginning of experiments. Vitamin-free casein, mineral mix and vitamin mix were obtained from Teklad (Madison, WI). Edible-grade cellulose, cornstarch, and sucrose were obtained from A.E.Staley (Decatur, IL). BBP (98%) was obtained from Aldrich Chemicals (Milwaukee, WI).

In experiments 1 and 2, the administration of BBP to rats for 5 days by i.p. injection or for 7 days by i.g. intubation did not result in significant differences in growth compared to controls (Table I). Treatment of animals with BBP by either route of administration was associated with a decrease in the formation of DMBA-DNA adducts in the mammary gland (Table I). The injection of 100 and 500 mg/kg doses of BBP was associated with a significant 72 and 91% inhibition, respectively, in the quantity of total mammary DMBA-DNA.

**Fig. 1.** Structure of benzyl butyl phthalate (BBP).

**Table I.** Effect of BBP administration on mammary DMBA-DNA adduct formation and liver GST and EROD activities

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Body wt (g)$^a$</th>
<th>DMBA-DNA adducts (nmol/DMBA/nmolDNA)</th>
<th>GST (nmol/min/mg)</th>
<th>EROD (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Anti-</td>
<td>Syn-</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>161.6 ± 6.3$^a$</td>
<td>739 ± 136$^a$</td>
<td>512 ± 98$^a$</td>
<td>180 ± 31$^a$</td>
</tr>
<tr>
<td>BBP (100 mg/kg, i.p.)</td>
<td>163.4 ± 5.1$^a$</td>
<td>205 ± 60$^b$</td>
<td>107 ± 39$^b$</td>
<td>70 ± 15$^b$</td>
</tr>
<tr>
<td>BBP (500 mg/kg, i.p.)</td>
<td>164.4 ± 3.5$^a$</td>
<td>68 ± 12$^b$</td>
<td>34 ± 7$^b$</td>
<td>25 ± 4$^b$</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>157.6 ± 3.0$^a$</td>
<td>400 ± 23$^a$</td>
<td>292 ± 20$^a$</td>
<td>71 ± 13$^a$</td>
</tr>
<tr>
<td>BBP (100 mg/kg, i.g.)</td>
<td>161.3 ± 5.4$^a$</td>
<td>300 ± 52$^{ab}$</td>
<td>226 ± 41$^{ab}$</td>
<td>46 ± 10$^a$</td>
</tr>
<tr>
<td>BBP (500 mg/kg, i.g.)</td>
<td>157.5 ± 4.3$^a$</td>
<td>209 ± 42$^{ab}$</td>
<td>146 ± 35$^{ab}$</td>
<td>43 ± 6$^a$</td>
</tr>
</tbody>
</table>

$^a$Values represent means ± SE. Body weights are for animals at end of BBP treatment period. Means among treatment groups within experiments sharing unlike superscripts are significantly different at $P<0.05$.

**Table II.** Effect of BBP administration on the initiation stage of DMBA-induced mammary tumorigenesis$^a$

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Body wt (g)</th>
<th>Final palpable tumor incidence (%)</th>
<th>Final palpable tumors per rat ($\bar{x}$)</th>
<th>Adenocarcinomas$^3$ per rat ($\bar{x}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>280.5 ± 5.4$^a$</td>
<td>82.1$^a$</td>
<td>3.1 ± 0.5$^a$</td>
<td>4.0 ± 0.6$^a$</td>
</tr>
<tr>
<td>BBP (250 mg/kg)</td>
<td>282.9 ± 6.1$^a$</td>
<td>51.9$^b$</td>
<td>1.3 ± 0.4$^b$</td>
<td>1.6 ± 0.5$^b$</td>
</tr>
<tr>
<td>BBP (500 mg/kg)</td>
<td>272.6 ± 4.0$^a$</td>
<td>51.9$^b$</td>
<td>0.9 ± 0.2$^b$</td>
<td>1.2 ± 0.3$^b$</td>
</tr>
</tbody>
</table>

$^a$Values represent means ± SE at week 15 of experiment 3. Means among treatment groups sharing unlike superscripts are significantly different at $P<0.05$.

$^3$Adenocarcinomas/rat values include both palpable and non-palpable tumors removed at necropsy. Only one tumor in each treatment group was identified as a fibroadenoma.
adducts. The formation of both anti- and syn-derived adducts was significantly depressed at both doses of BBP. When BBP was given i.g., there was a significant decrease in total mammary DMBA-DNA adducts only for those animals receiving the 500 mg/kg dose (Table I). BBP administered i.g. at 100 and 500 mg/kg doses was associated with a 25 and 48% decrease, respectively, in the quantity of total mammary adducts, compared to controls. The quantity of anti-, but not syn-derived adducts was significantly less for animals treated with 500 mg/kg BBP, compared to controls.

Treatment of animals with BBP (i.g.) did not significantly influence the liver activity of GST per mg protein, although there was an increase in liver GST activity for rats receiving both doses of BBP (Table I). The liver activity of EROD (a specific measure of cytochrome P4501A activity), was significantly increased following i.g. administration of BBP at 500 mg/kg (Table I). EROD activity per mg protein was increased by 2.3- and 8.5-fold for animals treated with BBP at doses of 100 and 500 mg/kg, respectively.

In experiment 3, administration of BBP to animals for 7 days prior to DMBA dosing did not result in significant differences in animal body weights among groups. After 7 days of BBP treatment (i.g.), body weights (x) were 161.6, 162.5 and 160.8 g for rats in the control, 250 mg/kg BBP and 500 mg/kg BBP groups, respectively. There was no significant difference in body weights among treatment groups during the 15 weeks following DMBA administration (Figure 2). DMBA-induced mammary tumorigenesis was suppressed by treatment of animals with BBP prior to DMBA administration (Figure 3, Table II). Comparison of tumor incidence curves by survival analysis, using the generalized Wilcoxon test statistic to determine equality of survivor functions, indicated that treatment with both doses of BBP was associated with a significant decrease in tumor incidence (control versus 250 mg/kg BBP, \( P = 0.014 \); control versus 500 mg/kg BBP, \( P = 0.009 \)). By 15 weeks post-DMBA, mammary tumor incidence was decreased by 37% for rats treated with both doses of BBP, compared to controls. The number of palpable tumors per rat at week 15 was significantly inhibited by 58 and 71%, for animals administered BBP at 250 and 500 mg/kg, respectively, compared to controls. In a similar manner, adenocarcinoma

Fig. 2. Body weight changes for animals in experiment 3.

Fig. 3. Effect of BBP treatment on DMBA-induced mammary tumorigenesis.
multiplicity decreased significantly by 60 and 70% for animals treated with BBP at 250 and 500 mg/kg doses, respectively, compared to controls.

The present report indicates that the environmental pollutant BBP, at the doses examined, acts as an inhibitor, not as an enhancer, of the initiation stage of mammary tumorigenesis induced by the PAH DMBA. The capacity of BBP to block mammary tumor initiation at the 500 mg/kg dose was associated with an inhibition in the in vivo formation of total mammary DMBA-DNA adducts. Part of the action of BBP at the 500 mg/kg dose in preventing DMBA-induced mammary DNA adduct formation and tumorigenesis may be explained by an increase in metabolism of DMBA in the liver, as suggested by the BBP-associated increase in liver EROD activity. No significant enhancement in the liver activity of the phase II detoxification enzyme GST was observed following administration of BBP. It should be noted, however, that the induction of Phase I enzyme activities could also result in an enhancement of carcinogenesis, particularly under conditions in which there is not a concomitant increase in Phase II enzyme activities. Therefore, the impact of BBP in other carcinogenesis models would need to be determined. In addition, the activities of other Phase I and Phase II enzymes involved in DMBA metabolism would need to be evaluated before the mechanisms of BBP inhibition of DNA adduct formation and tumorigenesis can be definitively established.

Our results are similar to others who have reported that an environmental pollutant (DDT) is capable of inhibiting the initiation of chemically-induced liver carcinogenesis (24). In these studies, it was observed that the onset of exposure to the pollutant was a critical factor in determining whether tumorigenesis was stimulated or inhibited. Another phthalate, di(2-ethylhexyl)phthalate (DEHP), has been reported to inhibit chemically-induced liver tumorigenesis (18,19), although the effect was noticed on the post-initiation stage. There are no other reports of phthalates acting as modulators of chemically-induced mammary carcinogenesis. The results of the present studies underscore the need to more thoroughly evaluate the effect of exposure to BBP and other phthalate plasticizers on the various stages of mammary carcinogenesis at environmentally relevant doses. Also, the impact of phthalate exposure on the activities of cytochromes P450 in the liver, mammary gland and other tissues, as well as on the metabolism of other mammary carcinogens (2) needs to be determined. These findings suggest that the effect of an environmental agent on the development of mammary cancer may depend on the specific carcinogen. Also, interactions between agents may be important to scrutinize, since it is likely that humans are exposed to a variety of pollutants capable of influencing breast cancer risk. Understanding the ultimate effect of environmental chemicals on human breast cancer risk may be best accomplished by evaluating the combined effect of several chemical and dietary factors (25). In the case of BBP, this phthalate plasticizer actually can act as a blocking agent toward PAH-induced mammary carcinogenesis, at least under the experimental conditions utilized here. Whether similar effects would be observed in human cells needs to be examined (26).

In summary, the plasticizer BBP, an ubiquitous environmental contaminant, was capable of inhibiting rat mammary carcinogenesis when administered prior to dosing with DMBA. The inhibition in mammary tumor formation was associated with a decrease in the in vivo formation of mammary DMBA-DNA adducts and an increase in liver activity of EROD. These results indicate that further evaluation of the effect of BBP and other phthalates on mammary tumorigenesis is warranted.

References

   Effect of varying the onset of exposure to DDT on its modulation of


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