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Enhancement of Butylated Hydroxytoluene-Induced Mouse Lung Damage by Butylated Hydroxyanisole[1]

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Enhancement of Butylated Hydroxytoluene-Induced Mouse Lung Damage by Butylated Hydroxytoluene. THOMPSON, D. C., AND TRUSH, M. A. (1988). *Toxicol. Appl. Pharmacol.* **96,** 115–121. The phenolic antioxidant butylated hydroxytoluene (BHT) is known to produce a dose-dependent increase in mouse lung weight which is characterized by the necrosis of pulmonary type I and endothelial cells. We studied the ability of butylated hydroxyanisole (BHA) to modify BHT-induced changes in lung weight in male CD-1 mice. BHA alone had no effect on lung weight up to a dose of 500 mg/kg (sc). However, when injected 30 minutes prior to subthreshold doses of BHT (0–250 mg/kg, ip), BHA significantly enhanced lung weight in a dose-dependent manner. The ability of BHA to enhance BHT-induced changes in lung weight was dependent on both the time and the route of administration of BHA relative to BHT. Deuteration of BHT abolished the *in vivo* toxicity from the combination of BHA and BHT. These results suggest that the toxicity resulting from the combination of BHA and BHT is due to the formation of BHT-quinone methide and that the role of BHA might be either to deplete some protective mechanism in the target pulmonary cells or to enhance the biotransformation of BHT into BHT-quinone methide.

One of the best-characterized toxic effects of butylated hydroxytoluene (BHT) is damage to type I pneumocytes in the mouse lung. The adverse effects of BHT in mouse lung were first established by Marino and Mitchell (1972). Detailed studies on the physiological and biochemical changes which occur after exposure to BHT as well as histologic and cell kinetic analyses have since been carried out (Witschi and Saheb, 1974; Saheb and Witschi, 1975; Adamson *et al.*, 1977; Witschi and Cote, 1977; Kuo *et al.*, 1978; Malkinson, 1979; Arany *et al.*, 1981; Smith, 1984; Malkinson *et al.*, 1985; Okine *et al.*, 1986). These studies have shown that the initial sites of damage are the capillary endothelial cells and the type I pneumocytes. Complete destruction of the type I cells occurs on Days 2 and 3 after BHT administration, after which a repair process that replaces the type I cells with differentiated type II cells is initiated. At low doses of BHT, pulmonary damage is completely reversible, whereas at higher doses excess collagen is deposited, which is characteristic of moderate pulmonary fibrosis.

The pulmonary toxicity of BHT is thought to be dependent on its cytochrome *P*-450-mediated metabolism to a reactive, electrophilic metabolite, BHT-quinone methide (Kehrer and Witschi, 1980; Mizutani *et al.*,

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1982, 1983). BHT–quinone methide can covalently bind to various cellular nucleophiles, especially those containing sulfydryl groups such as glutathione and cysteine (Nakagawa *et al.*, 1981; Tajima *et al.*, 1985). BHT–quinone methide has been detected *in vivo* in mouse liver and lung (Mizutani *et al.*, 1983).

We have recently observed that butylated hydroxyanisole (BHA) and other phenolic compounds can enhance both the *in vitro* peroxidative biotransformation of BHT into BHT–quinone methide and the covalent binding of BHT to microsomal protein (Thompson *et al.*, 1986, 1988). On the basis of these observations, we reasoned that BHA might also enhance the *in vivo* toxicity of BHT in mouse lung. BHA was chosen for these studies because it was the most effective enhancer of BHT–quinone methide formation *in vitro* and because it is often found in the same food and cosmetic products as BHT and thus their interaction might have some toxicologic relevance.

METHODS

Materials. BHA, BHT, lithium aluminum deuteride, and corn oil were obtained from Sigma (St. Louis, MO). BHT acid (3,5-di-*tert*-butyl-4-hydroxybenzoic acid) was purchased from Aldrich (Milwaukee, WI). Deuterated BHT was synthesized by the reduction of the methanol ester of BHT acid with lithium aluminum deuteride as described by Mizutani *et al.* (1983). The isotopic purity of the deuterated compound was greater than 99%. An *in vitro* assay for the formation of BHT–quinone methide using BHT and deuterated BHT indicated an isotope effect of 2.6 (Thompson and Trush, 1988a).

Mouse lung toxicity. Toxicity was assessed by measuring the changes in lung wet weight and body weight 4 days after the administration of BHT or BHA. Test compounds were administered by either subcutaneous (BHA) or intraperitoneal (BHT) injection using corn oil as the vehicle. Injection volumes were limited to 0.2 ml/30 g mouse (ip) or 0.1 ml/30 g mouse (sc). Corn oil itself had no effect on mouse lung weights. Male CD-1 mice (Charles River, Wilmington, MA), 4–5 weeks old, were used in all experiments. The mice were allowed a 2- to 3-day period for acclimation before use. Animals were allowed food (5002 Purina Certified Rodent Diet) and water *ad libitum* throughout the experiment, were housed on Alpha-Dry bedding (Metro Feeds, Ltd., Columbia, MD), and were kept on a 12-hr light/dark cycle in plastic cages. Mice were euthanized by spinal dislocation and the lungs excised and put into isotonic saline. The lungs were blotted dry and weighed. In these experiments normal (or corn oil control) lung weights were around 150 mg, whereas in animals administered 500 mg/kg of BHT the lung weights increased to approximately 350 mg due to BHT-induced lung edema. Body weights were also recorded. For control animals the body weights were generally around 25 g; however, in animals treated with 500 mg/kg of BHT, the body weights decreased to 21–22 g. A loss of body weight due to BHT administration is well documented (Malkinson, 1979; Mizutani *et al.*, 1982; Smith, 1984) and therefore our results are reported as lung/body weight ratios. Lung toxicity was confirmed by histological analysis of lungs from each treatment group. Lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin.

Statistical analyses. Statistical analyses were carried out following the guidelines outlined by Gad and Weil (1982). In general, the means and standard errors were calculated for all treatment groups. If the variances of all groups were homogeneous (Levene's test; Snedecor and Cochran, 1980), the data were subjected to analysis of variance followed by Duncan's multiple range test to determine which means were significantly different from each other or controls. If the variances of groups were heterogeneous, the data were converted to logarithms prior to the analysis of variance. This transformation effectively eliminated the heterogeneity. Student's *t* test was used to analyze the results in Table 1, which contained only three groups of data. In all cases a p value of <0.05 was used to determine significance.

RESULTS

The effects of various doses of BHT or BHA alone on mouse lung/body weight ratios are shown in Fig. 1. BHT alone had no effect on lung weight up to a dose of 175 mg/kg, but higher doses produced an increase in lung weight up to a dose of 500 mg/kg which was reflected in a significant increase in the lung/body weight ratio. Doses of BHT higher than 500 mg/kg did not further increase the lung/body weight ratio beyond a value of around 1.6% (not shown). Conversely, subcutaneous injections of BHA alone had no effect on lung weight up to a dose of 500 mg/kg. This mode of BHA administration was chosen to minimize any hepatic (Branen, 1975) or intestinal (Della Corte *et al.*, 1984) metabolism of BHA prior to its reaching the lung.

Doses at which BHT caused little or no alteration in lung weight were then used for the

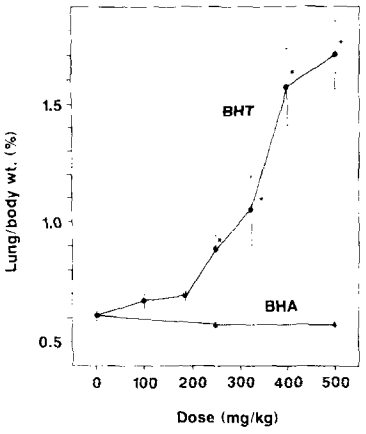

FIG. 1. Effect of BHA or BHT alone on mouse lung weight. Mice were given intraperitoneal (BHT) or subcutaneous (BHA) injections using corn oil as a vehicle. Points represent means ± standard error of at least 5 animals per dose. *Indicates points significantly different from corn oil control ($p < 0.05$).

experiments with BHA. These doses were 250 mg/kg and below. When BHA was injected 30 min prior to BHT, a dose-dependent enhancement of BHT-induced lung damage was observed (Fig. 2). At a dose of 250 mg/kg BHT, two BHA doses (50 and 250 mg/kg) produced a significant elevation of lung weight compared with BHT alone, while at the 175 mg/kg BHT dose, only the 250 mg/kg BHA dose caused a significant enhancement of lung weight. A trend toward enhancement was seen at the 175 mg/kg BHT dose with 50 mg/kg BHA and at the 100 mg/kg BHT dose with 250 mg/kg BHA, but these results were not statistically significant. Doses of less than 50 mg/kg BHA did not increase lung weight in animals given 250 mg/kg BHT (Fig. 3). Further experiments (described below) were carried out using 175 mg/kg BHT (rather than 250 mg/kg BHT) because administration of the higher dose sometimes had an effect on lung weight, while the lower dose always had no effect on lung weight. Thus, using 175 mg/kg of BHT in these combination experiments made it easier to distinguish the enhancing effects of BHA.

The enhancement of BHT-induced lung toxicity by BHA was influenced by both the time and the route of administration of BHA. BHA given simultaneously with BHT or up to 4 hr prior to BHT was able to significantly enhance lung weight (Fig. 4.). BHA given 2 hr after BHT also increased lung weight but the effect was not statistically significant. BHA given before or after these times had no

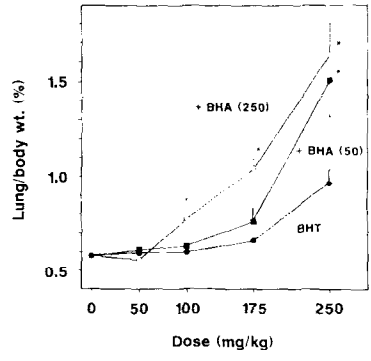

FIG. 2. Enhancement of BHT-induced mouse lung toxicity by BHA. Mice were given subcutaneous injections of BHA 30 min prior to intraperitoneal injections of BHT. (●) BHT alone; (■) BHT + 50 mg/kg BHA; (○) BHT + 250 mg/kg BHA. Points represent means ± standard of at least 5 animals. *Represents points which are significantly different from BHT alone ($p < 0.05$).

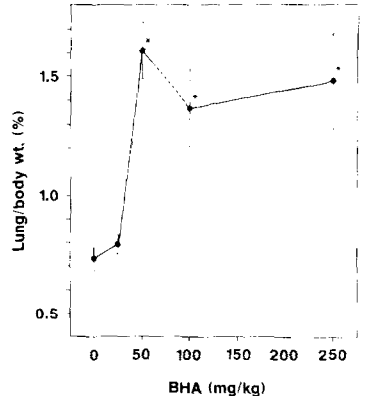

FIG. 3. Dose–response of BHA on BHT-induced mouse lung toxicity. Mice were given subcutaneous injections of various doses of BHA 30 min prior to an intraperitoneal injection of 250 mg/kg BHT. Points represent means ± standard error of at least 5 animals. *Represents points which are significantly different from BHT alone ($p < 0.05$).

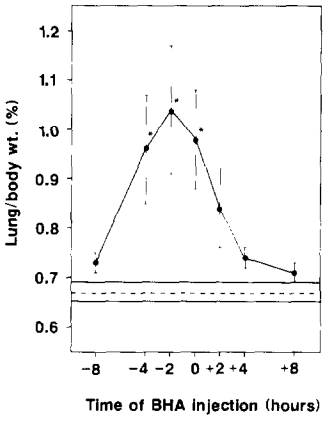

FIG. 4. Effect of time of administration of BHA on BHT-induced mouse lung toxicity. Mice were given subcutaneous injections of 250 mg/kg BHA at various times relative to an intraperitoneal injection of 175 mg/kg BHT. BHA was injected up to 8 hr prior to (−8 time point) or 8 h after (+8 time point) the BHT injection (represented as 0 hr). Points represent the means ± standard error of 14 animals per dose except for control (8 animals). The dashed line indicates control level (no BHA). *Represents points which are significantly different from BHT alone ($p < 0.05$).

effect on lung weight. When BHA was given by intraperitoneal injection, an enhancing effect was observed (Table 1) but it was less dramatic than when given by subcutaneous injection.

We also tested the ability of *tert*-butylhydroquinone (TBHQ), a cytochrome *P*-450-mediated microsomal metabolite of BHA, to enhance BHT toxicity (Table 2). At a dose of 62.5 mg/kg, TBHQ had no effect on lung weight by itself or in combination with 175 mg/kg BHT. The dose of TBHQ tested was based on the highest nonlethal dose given CD-1 mice by Krasavage and O'Donoghue (1984).

Deuterium substitution has been a useful chemical tool in determining metabolic pathways and in identifying possible toxic metabolites (Klinman, 1978; Pohl and Gillette, 1984-5). Mizutani *et al.* (1983) used deuterated BHT to suggest that the toxic metabolite responsible for eliciting mouse lung damage is BHT–quinone methide. We synthesized deuterated BHT in order to determine whether the lung damage seen in experiments with the combination of BHA and BHT was caused by BHT–quinone methide. We observed an *in vivo* isotope effect which is shown in Table 3. Deuterated BHT had no pulmonary toxic effect at 175 mg/kg and was significantly less toxic (1.16 ± 0.10) at a dose of 400 mg/kg than was a corresponding dose of BHT (1.69 ± 0.14). These results confirm the observations of Mizutani *et al.* (1983). In the presence of 250 mg/kg BHA, deuterated BHT was also significantly less toxic (0.70 ± 0.07) than BHT (1.06 ± 0.19). These results suggest that the lung damage which results from combinations of BHA and BHT is probably caused by the same metabolite, BHT–quinone methide, which is responsible

TABLE 1

COMPARISON OF ROUTES OF ADMINISTRATION OF BHA ON BHT-INDUCED MOUSE LUNG TOXICITY

Group	Lung/body wt (%)	*N*
Control	0.58 ± 0.03	5
BHT 175/BHA 250 (sc)	1.01 ± 0.17*	10
BHT 175/BHA 250 (ip)	0.80 ± 0.13	10

Note. Mice were injected with 250 mg/kg body wt of BHA (subcutaneous [sc] or intraperitoneal [ip]) 30 min prior to an ip injection of 175 mg/kg BHT. Values represent mean ± standard error. *N* = number of animals.

* Represents values which are significantly different from control (Student *t* test, $p < 0.05$).

TABLE 2

EFFECT OF *tert*-BUTYLHYDROQUINONE (TBHQ) ON BHT-INDUCED MOUSE LUNG TOXICITY

Group	Lung/body wt (%)	*N*
Control—corn oil	0.56 ± 0.02	5
TBHQ (62.5)	0.56 ± 0.02	5
BHT (175)	0.60 ± 0.03	5
TBHQ/BHT	0.62 ± 0.02	10

Note. TBHQ (62.5 mg/kg, subcutaneous injection) or BHT (175 mg/kg, intraperitoneal injection) or both were administered to mice in corn oil. Values represent mean ± standard error. *N* = number of animals.

TABLE 3

In Vivo Isotope Effects of Deuterated BHT

Group	Lung/body wt (%)	*N*
Control—corn oil	0.59 ± 0.02	4
BHT 175	0.62 ± 0.02	10
d_3-BHT 175	0.60 ± 0.01	10
BHT 400	1.69 ± 0.14*	10
d_3-BHT 400	1.16 ± 0.10*,**	10
BHT 175/BHA 250	1.06 ± 0.19*	10
d_3-BHT 175/BHA 250	0.70 ± 0.07***	10

Note. Mice were given ip injections of 175 or 400 mg/kg BHT or deuterated BHT (d_3-BHT). BHA (250 mg/kg) was administered by sc injection 30 min prior to BHT or d_3-BHT. Values represent mean ± standard error. *N* = number of animals.

* Denotes values which are significantly different from corn oil control.

** Denotes values which are significantly different from BHT 400.

*** Denotes values which are significantly different from BHT 175/BHA 250 ($p < 0.05$).

for lung toxicity resulting from high doses of BHT alone.

DISCUSSION

Our results clearly demonstrate that BHA can potentiate the murine pulmonary toxicity induced by the ip administration of BHT. Since BHA does not appear to have any pulmonary toxicity of its own (Fig. 1), this interaction cannot be considered additive. Previous reports have similarly indicated that BHA and other antioxidants (with the exception of BHT) do not cause lung damage (Larsen and Tarding, 1978; Malkinson, 1979; Krasavage and O'Donoghue, 1984). Mizutani *et al.* (1982) have suggested through structure–activity studies that the reason these compounds are not pulmonary toxins is because of their inability to form quinone methides. Similarly, our results with deuterated BHT (Table 3) further indicate that the pulmonary damage caused by the combination of BHT and BHA was due to the formation of BHT–quinone methide from BHT.

The data presented above demonstrated several requirements for the potentiation of BHT-induced lung damage by BHA. First, the route of administration of BHA had an influence on the amount of damage observed (Table 1). BHA given by subcutaneous injection always produced a greater amount of toxicity than when it was given by intraperitoneal injection. This might be due to the fact that a larger portion of an intraperitoneal BHA dose would go directly to the liver, where BHA would undergo detoxication and conjugation reactions and result in a smaller quantity of parent BHA reaching the lung intact. This also suggests that the interaction between BHA and BHT occurs in the lung. Second, the time of administration of BHA relative to BHT also influenced the amount of toxicity observed (Fig. 4). It was necessary to give BHA concurrent with or shortly before BHT in order to see any effect. And third, TBHQ, a major metabolite of BHA, had no enhancing effect on BHT-induced lung toxicity. This suggests that the parent BHA molecule is necessary for the effect to be observed. Taken together, these three observations strongly suggest that the parent BHA molecule must be present in the lung at approximately the same time as BHT for an enhancing effect to be seen. It has recently been reported that BHA and TBHQ stimulate the formation of hydrogen peroxide *in vitro* in rat liver microsomes (Cummings and Prough, 1983; Rossing *et al.*, 1985). If this reaction of TBHQ occurs *in vivo*, our data suggest that increased formation of hydrogen peroxide is not, in and of itself, sufficient to increase BHT-induced toxicity.

Although the exact mechanism(s) by which BHA increases the toxicity of BHT is unknown at this time, several possibilities exist. One such mechanism is that BHA interferes with the kinetics of BHT absorption or metabolism in the liver and allows more parent BHT to reach the lung, thus increasing the amount of toxic metabolite formed. We feel this suggestion is unlikely, however, since our choice of a subcutaneous route of administration for BHA minimized this possibility and

the fact that subcutaneous administration of BHA was more effective than its intraperitoneal administration. A second possible mechanism is that BHA interferes with the repair process initiated post-BHT damage. This is also unlikely since our results show that if BHA is given 4 hr after BHT no enhancing effect was observed. Two other possibilities stand out as the most likely in view of the results presented above. These are either that BHA interferes with a protective mechanism in target lung cells, such as by depleting glutathione levels, or that BHA increases the metabolic activation of BHT in the lung. This latter possibility is of particular interest in light of recent observations from our laboratory. We have observed that BHA, but not TBHQ, enhances the covalent binding of BHT to protein and also the formation of BHT–quinone methide in the presence of peroxidase enzymes (Thompson and Trush, 1988a). Further studies on the possible mechanism of enhancement of BHT toxicity by BHA will be presented in the following paper (Thompson and Trush, 1988b).

Our results demonstrate a potentially toxic interaction between two commonly used food antioxidants. This is of interest from a toxicologic standpoint since these compounds are ingested in substantial quantities by humans (Gosselin *et al.*, 1984). Although BHT-induced pulmonary toxicity is reversible and occurs only at high doses in mice, we have shown that BHA can significantly lower the threshold for BHT-induced lung toxicity. In addition to lung toxicity, BHT has been shown to cause hemorrhagic death in rats (Takahashi and Hiraga, 1978) and to enhance tumor growth in various tissues of both mice and rats (Witschi, 1985; Imaida *et al.*, 1984). Although it has not been established what role BHT–quinone methide may play, if any, in these other BHT-induced toxicities, it would be of interest to see if BHA affects these conditions as well. Numerous synergistic or additive toxic effects from combinations of various drugs or chemicals are known (Murad and Gilman, 1985), but the toxic effects of combinations of antioxidants have not been well studied. An interesting recent report by Hirose *et al.* (1986) demonstrated that propyl gallate and ethoxyquin enhanced BHA-induced epithelial hyperplasia in rat forestomach. Our results suggest that possible toxic interactions of BHT with BHA and other antioxidants should be examined.

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