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Studies on the Mechanism of Enhancement of Butylated Hydroxytoluene-Induced Mouse Lung Toxicity by Butylated Hydroxyanisole[1]

DAVID C. THOMPSON[2] AND MICHAEL A. TRUSH

Department of Environmental Health Sciences, Division of Toxicological Sciences, Johns Hopkins University, Baltimore, Maryland 21205

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Studies on the Mechanism of Enhancement of Butylated Hydroxytoluene-Induced Mouse Lung Toxicity by Butylated Hydroxyanisole. THOMPSON, D. C., AND TRUSH, M. A. (1988). *Toxicol. Appl. Pharmacol.* **96,** 122–131. The studies described in this report were designed to probe possible mechanisms whereby butylated hydroxyanisole (BHA) is able to enhance butylated hydroxytoluene (BHT)-induced mouse lung toxicity. In experiments with mouse lung slices, BHA enhanced the covalent binding of BHT to protein, indicating that the interaction between BHA and BHT takes place in the lung. Subcutaneous administration of either BHA (250 mg/kg) or diethyl maleate (DEM, 1 ml/kg) to male CD-1 mice produced a similar enhancement of BHT-induced lung toxicity. In contrast to DEM, the administration of BHA (250 or 1500 mg/kg) did not decrease mouse lung glutathione levels, suggesting that the effect of BHA is not due to the depletion of glutathione levels. We previously observed that in the presence of model peroxidases a unique interaction occurs between BHA and BHT, resulting in the increased metabolic activation of BHT. Upon the addition of hydrogen peroxide or various hydroperoxides to mouse lung microsomes, BHA significantly increased the covalent binding of BHT to protein. BHA also stimulated the rate of formation of hydrogen peroxide by 4.7-fold in mouse lung microsomes. Likewise, hydrogen peroxide resulting from the NADPH cytochrome *P*-450 (*c*) reductase-catalyzed redox cycling of *tert*-butylhydroquinone, a microsomal metabolite of BHA, supported the peroxidase-dependent BHA-enhanced formation of BHT-quinone methide. These results suggest that BHA could facilitate the activation of BHT in the lung as a result of both the increased formation of hydrogen peroxide and the subsequent peroxidase-dependent formation of BHT-quinone methide from the direct interaction of BHA with BHT.

Several compounds have been shown to be capable of altering the pulmonary toxicity elicited by butylated hydroxytoluene (BHT) in mice. These compounds fall generally into three categories: (1) substances which alter cytochrome *P*-450 activity, including phenobarbital (Williamson *et al.*, 1978), piperonyl butoxide, and SKF 525-A (Kehrer and Witschi, 1980), cedrene (Malkinson, 1979), carbon disulfide, and diethyldithiocarbamate (Masuda and Nakayama, 1984); (2) substances which interfere wtih the repair process initiated following BHT-induced damage such as hyperbaric oxygen (Haschek and Witschi, 1979) radiation (Haschek *et al.*, 1980), 1,3-bis-(2-chloroethyl)-1-nitrosourea (Kehrer and Klein-Szanto, 1985), or corticosteroids (Hakkinen *et al.*, 1983; Kehrer *et al.*, 1984; Okine *et al.*, 1986); and (3) substances

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[2] To whom correspondence should be addressed at current address: National Institute of Environmental Health Sciences, Laboratory of Molecular Biophysics (MD 14-03), PO Box 12233, Research Triangle Park, NC 27709.

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which alter the *in vivo* reduced thiol status. This latter group includes compounds such as diethyl maleate and buthionine sulfoximine, which enhance BHT-induced pulmonary toxicity, and cysteine, which protects against damage (Mizutani *et al.*, 1984). Of the compounds which enhance BHT pulmonary toxicity, however, none are thought to do so by increasing the formation of the purported toxic metabolite of BHT, BHT-quinone methide.

In the previous manuscript (Thompson and Trush, 1988a) we reported that subcutaneous administration of BHA is able to enhance mouse lung toxicity elicited by subthreshold doses of BHT. In this report we extend our studies to probe possible mechanisms underlying the toxicological interaction resulting from the administration of these two antioxidants and have considered two possibilities: (a) BHA depletes critical protective nucleophiles (such as glutathione), rendering the target lung cells more susceptible to damage from BHT-derived metabolites, and (b) BHA enhances the metabolic activation of BHT, resulting in the increased formation of BHT-quinone methide.

METHODS

Materials. BHA, BHT, diethyl maleate, glutathione, horseradish peroxidase (Type II), hydrogen peroxide (30% solution), and cumene hydroperoxide were obtained from Sigma (St. Louis, MO). *tert*-Butylhydroquinone (TBHQ), benzoyl peroxide, potassium cyanide, sodium azide, and *tert*-butyl peroxide were obtained from Aldrich (Milwaukee, WI). NADPH cytochrome *P*-450 (*c*) reductase (purified from phenobarbital treated rat liver) was a generous gift of Dr. Hirokata of the National Institutes of Health. Hydroperoxy-BHT (2,6-di-*tert*-butyl-4-hydroperoxy-4-methyl-2,5-cyclohexadienone) was prepared by the method of Kharasch and Joshi (1957). [^{14}C]BHT (20 mCi/mmol) was purchased from Amersham (Arlington Heighs, IL).

Mouse lung toxicity. Toxicity was measured as change in lung wet weight and body weight 4 days after the administration of BHT and/or test compounds. All compounds were dissolved in corn oil and injected in a volume not exceeding 0.2 ml/30 g mouse (intraperitoneal) or 0.1 ml/30 g mouse (subcutaneous). Male CD-1 mice (Charles River, Wilmington, MA), 4–5 weeks old, were used in all experiments. Other conditions were as previously described (Thompson and Trush, 1988).

Covalent binding of BHT to protein. Incubation conditions consisted of 1 mg of mouse lung microsomal protein, 100 μM (0.25 μCi) BHT, and 1 mM sodium azide in 1 ml of 0.1 M Tris buffer (pH 7.5). In some reactions 100 μM BHA was also present. BHA and BHT were added in 10 μl DMSO. The reactions were initiated by the addition of varying concentrations of hydrogen peroxide or organic peroxides and incubated at 37° for 10 min. Reactions were stopped by the addition of 4 ml methanol and centrifuged at 2300 rpm for 10 min to pellet the protein. The protein pellets were subsequently washed with methanol and methanol/ether (3/1) until no further radioactivity could be extracted. The protein pellets were redissolved in 1 ml of 1 N NaOH with heating at 60° for 1 hr. A 0.5-ml aliquot was added to 10 ml ACS (Amersham) counting scintillant and the radioactivity measured on a Packard Tri-Carb 300 liquid scintillation counter. Another aliquot (50 μl) was used for protein measurement by the Lowry method (Lowry *et al.*, 1951).

Glutatione measurement. Glutathione from mouse lungs was assayed using two separate procedures. Reduced glutathione was measured following the procedure of Ellman (1959). Oxidized glutathione (GSSG) was determined by the method of Allison and Shoup (1983) using HPLC and two electrochemical detectors. Lung samples were homogenized in 5 ml of 0.05 M perchloric acid and centrifuged at 12,000*g* for 2 min. Supernatant (200 μl) was reacted with 300 μl of 0.24 M *N*-ethylmaleimide for 30 min at room temperature. The sample was diluted at 1 ml with acid and 20 μl was injected onto the HPLC column for GSSG assay.

NADPH oxidation. NADPH oxidation was measured according to the procedure of Cummings and Prough (1983). Liver microsomes were prepared from mice pretreated with phenobarbital (100 mg/kg) for 3 days in order to induce cytochrome *P*-450 enzymes. Lung microsomes were prepared from untreated mice. Reactions contained 0.25 (liver) or 0.35 (lung) mg microsomal protein in 1 ml of 0.1 M Tris buffer, pH 7.5. Incubations were carried out at 37°C and were initiated by the addition of 180 μM NADPH. BHA or TBHQ was added 5 min later and the oxidation of NADPH was monitored by following the loss of absorbance at 340 nm. An extinction coefficient of 6220 M^{-1} cm^{-1} was used to determine NADPH concentrations (Klingenberg, 1981). For the reactions with a lag phase, the rates were obtained by constructing a line tangent to the maximal slope.

Hydrogen peroxide formation. Hydrogen peroxide formation was measured using the ferroammonium sulfate/potassium thiocyanate method as described by Hildebrandt *et al.* (1978). Reactions contained 1 mg/ml microsomal liver or lung protein, 1 mM NADPH, and 1 mM sodium azide (to inhibit catalase) in 1 ml of 0.1 M Tris buffer, pH 7.5. BHA or TBHQ was added after 5 min.

After incubation at 37°C for varying lengths of time, the reactions were terminated by the addition of 1 ml of ice-cold 10% trichloroacetic acid. The precipitated samples were centrifuged for 10 min at 22,000*g* and the supernatants assayed for hydrogen peroxide. Concentrations of hydrogen peroxide were estimated from a standard curve. The rates of hydrogen peroxide formation were determined by calculating the maximum rate of increase in hydrogen peroxide concentration between two time points (where the rate did not change over at least three consecutive points).

Formation of BHT-quinone methide. A complete reaction mixture contained 15 μg purified cytochrome *P*-450 (*c*) reductase, 200 μM NADPH, and 50 μM TBHQ in 1 ml of 0.1 M Tris buffer, pH 7.5. After incubation at 37°C for 10 min, 100 μM BHA, 200 μM BHT, and 7.5 units horseradish peroxidase (100 μg) were added. The formation of BHT-quinone methide was monitored spectrophotometrically at 300 nm as previously described (Thompson *et al.*, 1988).

Mouse lung slices. Lung slice incubations were carried out following the method of Fakjian and Buckpitt (1984). Fourteen male CD-1 mice were pretreated with diethyl maleate (1 ml/kg, sc) and euthanized by cervical dislocation 4 hr later. The lungs were removed, sliced with a razor blade into cubes of 0.5 to 1.0 mm, and placed in a beaker on ice containing 0.02 M Hepes-buffered balanced salt solution, pH 7.4. The buffer contained (in addition to 0.02 M Hepes) 140 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 5 mM NaOH, and 5.5 mM glucose (added the day of the experiment). Oxygen was continuously bubbled through the Hepes buffer containing the lung slices until the experimental preparations were complete. Approximately 150 mg of lung slices was added to glass vials containing 3 ml of the Hepes buffer and bubbled with oxygen for an additional 20 sec, 1 mM BHA or vehicle (DMSO) was added, and the vials were capped and incubated at 37°C for 60 min. At the end of 60 min, 1 mM BHT (containing 0.5 μCi ^{14}C-labeled BHT) was added and the vials were incubated an additional 90 mins. At the end of the second incubation period, the vials were transferred to an ice bath, the contents of each were homogenized, and 1 ml of 10% TCA was added to precipitate the protein. Covalent binding of BHT to lung protein was determined as described above. Heat-inactivated lung slices (autoclaved 30 min) were used in control incubations for both groups.

Statistical analyses. Where indicated, the data were subjected to analysis of variance followed by Duncan's multiple range test to determine significant differences between means. A p value of <0.05 was used to determine significance.

RESULTS

To confirm our underlying hypothesis that the interaction of BHA with BHT which results in increased pulmonary toxicity occurs *in situ* in the lung, we investigated the effect of BHA on the covalent binding of BHT in mouse lung slices. The results of this experiment are shown in Table 1. This experiment was carried out using glutathione-depleted lung slices in order to optimize the covalent binding of BHT. Heat-inactivated lung slices were used as controls for both groups. In the absence of any exogenous substrate, BHA was observed to enhance the covalent binding of BHT by 360%. The magnitude of stimulation of covalent binding was similar to what we have previously observed in *in vitro* peroxidase-catalyzed reactions (400%, Thompson *et al.*, 1988).

We next considered the possibility that BHA is itself activated to a reactive intermediate and as such depletes glutathione or other critical thiols, thus making the target lung cells more susceptible to the toxic effects of BHT. Diethyl maleate (DEM) is known to chemically deplete reduced glutathione levels *in vivo* (Plummer *et al.*, 1981). BHT has also been shown to decrease glutathione levels in rat liver and mouse lung and the intraperitoneal injection of DEM was observed to enhance BHT lung toxicity when given 1 hr

TABLE 1

ENHANCEMENT OF BHT COVALENT BINDING IN MOUSE LUNG SLICES BY BHA[a]

Reaction	nmol BHT bound/mg protein/90 min
BHT	0.10 ± 0.02
BHT and BHA	0.36 ± 0.04

[a] Lung slices were prepared from mice pretreated with diethyl maleate 4 hr prior to the preparation of the lung slices. Lung slices were incubated at 37°C for 60 min in the presence of 1 mM BHA or vehicle (DMSO). After this time period, 1 mM (0.5 μCi) [^{14}C]BHT was added and the reactions were incubated an additional 90 min. Heat-inactivated lung slices (autoclaved 30 min) were used in control incubations for both groups and these values (0.45 nmol for BHT, 0.62 nmol for BHT and BHA) were then subtracted from the results obtained using normal lung slices to obtain final values listed above. Values represent means ± standard error of triplicate reactions.

TABLE 2

COMPARISON OF THE EFFECT OF DIETHYL MALEATE OR BHA ON BHT-INDUCED MOUSE LUNG TOXICITY

Group	Lung/body wt (%)	N
Control—corn oil	0.56 ± 0.02	4
BHT (175)	0.55 ± 0.02	4
BHA (250)	0.57 ± 0.01	4
DEM	0.54 ± 0.02	4
BHT 175/BHA 250	1.04 ± 0.15*	9
BHT 175/DEM	1.06 ± 0.15*	9

Note. Diethyl maleate (DEM, 1 ml/kg) or BHA (250 mg/kg) was given by subcutaneous injection in corn oil 30 min prior to an intraperitoneal injection of BHT (175 mg/kg). Values represent means ± standard error. *N* = number of animals.

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

post-BHT (Nakagawa *et al.*, 1984; Mizutani *et al.*, 1984). Thus, we compared the ability of DEM (injected subcutaneously) to enhance BHT lung toxicity to that occurring with BHA (Table 2). At a dose of 1 ml/kg, DEM had no effect on mouse lung weight. However, when DEM was administered with a dose of 175 mg/kg BHT, a significant increase in lung weight was observed. The magnitude matched almost exactly the increase elicited by the combination of BHT with 250 mg/kg BHA. These results suggested that depletion of glutathione by BHA was a plausible mechanism for the enhancement of BHT toxicity. However, when the levels of glutathione in mouse lung tissue were measured following treatment with either DEM or BHA, only DEM caused a significant reduction in the level of reduced glutathione (Table 3). Even a high dose of BHA (1500 mg/kg, six times the amount which increases BHT lung toxicity) had no apparent effect on glutathione levels. In a separate experiment, oxidized levels of glutathione (measured by HPLC with electrochemical detection) in mouse lung were also unchanged by treatment with 250 mg/kg BHA (0.022 ± .005 vs 0.026 ± .008 μmol GSSG/g lung for control and BHA treated, respectively). Thus, while depletion of glutathione can enhance BHT toxicity, it seems unlikely that this is the mechanism for the enhancement of BHT lung toxicity by BHA.

We next turned our attention to the possibility that BHA might be able to increase the metabolic activation of BHT. We had previously observed that BHA enhanced BHT activation catalyzed by model peroxidase enzymes (Thompson *et al.*, 1986). We thus examined whether peroxidase enzymes capable of activating BHT to a covalently bound product were present in mouse lung. We observed no covalent binding of BHT to lung microsomal protein in the presence or absence of BHA when arachidonic acid was used as a peroxidase substrate. However, we did observe significant binding of BHT in the presence of hydrogen peroxide (Fig. 1). This binding was dependent on the concentration of peroxide and was enhanced by approximately 250% in the presence of 100 μM BHA. This suggested that a peroxidase other than prostaglandin H synthase was responsible for the covalent binding of BHT in the lung microsomes. Other organic peroxides, including cumeme hydroperoxide, *tert*-butyl peroxide, benzoyl peroxide, and hydroperoxy-BHT, were also able to support this binding (Table 4). Inhibitors of cytochrome *P*-450, such as

TABLE 3

COMPARISON OF THE EFFECT OF DIETHYL MALEATE OR BHA ON MOUSE LUNG GLUTATHIONE LEVELS

Group	μmol GSH/g lung	% of control
Control	2.45 ± 0.32	100
DEM	0.43 ± 0.06*	18
BHA 250	2.23 ± 0.26	91
BHA 1500	2.81 ± 0.13	115

Note. Diethyl maleate (DEM, 1 ml/kg) or BHA (250 or 1500 mg/kg) was administered by subcutaneous injection into mice 4 hr prior to the time of analysis. Control animals received corn oil. Values represent means ± standard error of 5 animals per group.

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

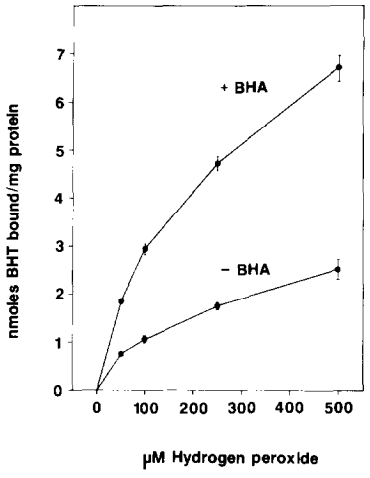

FIG. 1. Hydrogen peroxide-dependent covalent binding of BHT to mouse lung microsomes. Reactions contained 100 μM (0.25 μCi) BHT, ±100 μM BHA, 1 mg mouse lung microsomes, and varying concentrations of hydrogen peroxide in 1 ml of 0.1 M Tris buffer (pH 7.5). Points represent means ± standard error of triplicate reactions.

SKF 525-A and cyanide, as well as heat inactivation, inhibited the cumene hydroperoxide-dependent covalent binding of BHT in mouse lung microsomes. This suggested that the binding of BHT is enzyme mediated and that most likely it is catalyzed via the peroxidase activity of cytochrome *P*-450 (Hrycay and O'Brien, 1971).

BHA has been shown to increase the formation of hydrogen peroxide in microsomes isolated from rats, guinea pigs, and hamsters (Rossing *et al.*, 1985). Cummings and Prough (1983) suggested that the cytochrome *P*-450-dependent formation of TBHQ from BHA and its subsequent oxidation and redox cycling were responsible for the increased formation of hydrogen peroxide. To test this hypothesis, we measured the effect of BHA and TBHQ on the rate of oxidation of NADPH and the effect of BHA on the rate of formation of hydrogen peroxide by mouse liver and lung microsomes. Figure 2 illustrates a typical experiment measuring NADPH oxidation by mouse lung microsomes. The addition of increasing concentrations of BHA (traces B–D) resulted in increased rates of NADPH oxidation. Trace E represents the rate in the presence of TBHQ. The lag period between the addition of BHA and the resultant increase in the rate of NADPH oxidation has been attributed to the time necessary to metabolize BHA to TBHQ (Cummings and Prough, 1983; Cummings *et al.*, 1985).

The influence of BHA on the rates of NADPH oxidation and hydrogen peroxide formation in mouse liver and lung microsomes is shown in Table 5. A 3.6-fold increase in NADPH oxidation was accompanied by a 3.5-fold increase in hydrogen peroxide formation in liver microsomes, whereas

TABLE 4

PEROXIDE-DEPENDENT COVALENT BINDING OF BHT TO MOUSE LUNG MICROSOMES

Reaction	nmol BHT bound/ mg protein
A. Hydroperoxide-dependent covalent binding of BHT	
Hydrogen peroxide	8.88 ± .54
t-Butyl peroxide	5.18 ± .48
Cumene hydroperoxide	10.54 ± .40
Benzoyl peroxide	14.44 ± .66
Hydroperoxy-BHT	17.53 ± 1.73
B. Inhibition of cumene hydroperoxide-dependent covalent binding of BHT	
Cumene hydroperoxide	10.65 (100)
+SKF 525-A	9.10 (85)
+Cyanide	4.05 (38)
Heat-inactivated microsomes	0.96 (9)

Note. In section A, reactions were initiated by the addition of 1 mM hydrogen peroxide (or the indicated peroxide) to a reaction mixture containing 100 μM BHA, 100 μM (0.25 μCi) [^{14}C]BHT, 1 mM sodium azide, and 1 mg mouse lung microsomal protein in 1 ml of 0.1 M Tris buffer (pH 7.5). Reactions were incubated for 10 min at 37°C. Values in section A represent means ± standard error of at least 3 replicate incubations. In section B, reaction conditions were the same except that all reactions were initiated with 1 mM cumene hydroperoxide. The concentration of SKF 525-A and cyanide was 1 mM. Microsomes were heat inactivated by autoclaving for 30 min. Values in section B represent means of two reactions, while the number in parentheses indicates percentage of cumene hydroperoxide reaction.

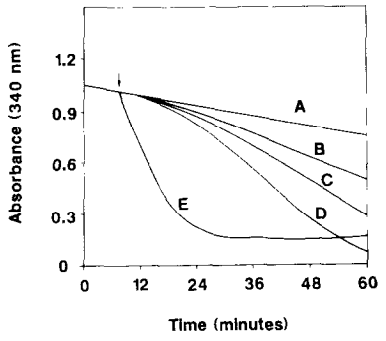

FIG. 2. Enhancement of the rate of NADPH oxidation in mouse lung microsomes by BHA or TBHQ. Reactions contained 200 μM NADPH, 0.2 mg mouse lung microsomes, and varying concentrations of BHA or TBHQ (added at 6 min—arrow) in 1 ml of 0.1 M Tris buffer (pH 7.5). (A) Endogenous rate of NADPH oxidation (no BHA or TBHQ); (B) 100 μM BHA; (C) 200 μM BHA; (D) 500 μM BHA; (E) 100 μM TBHQ.

there were 4.6- and 4.7-fold increases in these same two parameters with the lung microsomes. These results demonstrate that BHA, presumably through the formation of TBHQ, can increase the concentration of hydrogen peroxide in mouse lung microsomes.

Finally, we measured the ability of NADPH cytochrome *P*-450 (*c*) reductase to initiate the redox cycling of TBHQ and TBQ which would result in the formation of hydrogen peroxide. This hydrogen peroxide could subsequently be used to catalyze the peroxidative metabolism of BHT. Using purified cytochrome *P*-450 (*c*) reductase, TBHQ, and NADPH, a sufficient quantity of hydrogen peroxide was generated to serve as substrate for the horseradish peroxidase-dependent formation of BHT–quinone methide (Table 6). If any of the reaction components was omitted, no BHT–quinone methide was detected.

DISCUSSION

In the previous report (Thompson and Trush, 1988a), we reported that BHA increases the toxic effects of BHT in the mouse lung. In the present report, we have demonstrated that BHA can enhance the covalent binding of BHT in mouse lung slices, thus establishing that the interaction between these two antioxidants can occur directly in the lung. This interaction appears to be enzyme mediated since heating the lung slices prior to incubation decreases the covalent binding of BHT in the presence or absence of BHA. Experiments with lung microsomes indicated that there is a peroxidase present in mouse lung which is capable of activating BHT. The

TABLE 5

NADPH OXIDASE ACTIVITY AND HYDROGEN PEROXIDE FORMATION OF MOUSE LIVER AND LUNG MICROSOMES IN THE PRESENCE OF BHA

	Endogenous rate	BHA-stimulated rate[a]	Fold increase
		nmol/min/mg protein	
Liver			
NADPH oxidation	25 ± 0.4	89 ± 0.7	3.6
Hydrogen peroxide formation	18 ± 0.9	63 ± 2.5	3.5
Lung			
NADPH oxidation	3.9 ± 0.2	18 ± 1.4	4.6
Hydrogen peroxide formation	0.8 ± .03	3.9 ± 0.3	4.7

[a] BHA concentration in liver microsomal incubations was 100 μM while in lung microsomal incubations it was 500 μM. These concentrations were determined to be maximally stimulatory. Values represent means ± standard error of triplicate reactions.

TABLE 6

TBHQ-DEPENDENT FORMATION OF BHT-QUINONE METHIDE (QM)

Reaction	nmol QM formed/min
Complete[a]	26.7 ± 2.1
−TBHQ	n.d.
−BHA	n.d.
−BHT	n.d.
−Cytochrome *P*-450 (*c*) reductase	n.d.
−Horseradish peroxidase	n.d.
−NADPH	n.d.

[a] Complete reaction contained 15 μg cytochrome *P*-450 (*c*) reductase (purified from phenobarbital-treated rat liver), 200 μM NADPH, and 50 μM TBHQ. After incubation for 10 min, 100 μM BHA, 200 μM BHT, and 7.5 units horseradish peroxidase were added. The formation of BHT-quinone methide was monitored at 300 nm. n.d., not detected.

activity of this peroxidase with regard to BHT is sensitive to the presence of BHA. The observation that various organic peroxides as well as hydrogen peroxide, but not arachidonic acid, support the activation of BHT suggests that the enzyme responsible is the peroxidase activity of cytochrome *P*-450.

Concerning possible mechanisms for the stimulation of BHT-induced lung toxicity by BHA, the depletion of GSH by BHA does not appear to be involved. The formation of a BHA-GSH conjugate from microsomal incubations has recently been described by Cummings *et al.* (1985). However, even if this conjugate is formed *in vivo*, it does not appear to play a significant role in increasing BHT toxicity since even very large doses of BHA (1500 mg/kg) do not lower pulmonary GSH levels. However, the alternative mechanism, that BHA may increase the metabolism of BHT to BHT-quinone methide through a peroxidase-catalyzed mechanism, seems to be supported by our data. This mechanism is illustrated in the diagram shown in Fig. 3. In this mechanism BHA serves a dual function. First, BHA serves as a source of hydrogen peroxide as a result of its metabolism by cytochrome *P*-450 to form TBHQ, which then autooxidizes to form TBQ. The reduction of TBQ to the semiquinone by NADPH-cytochrome *P*-450 (*c*) reductase results in the interaction of the semiquinone with oxygen, generating superoxide anions. This continuing redox cycling leads to the further formation and buildup of hydrogen peroxide as a result of the dismutation of superoxide anions. Second, BHA also serves to enhance the hydrogen peroxide-dependent oxidation of BHT, resulting in the formation of BHT-quinone methide. The shift to the peroxidative mode of catalytic activity of cytochrome *P*-450 is favored by the shuttling of electrons away from cytochrome *P*-450 as a result of the NADPH-cytochrome *P*-450 (*c*) reductase-catalyzed redox cycling of TBHQ and TBQ. The shuttling of electrons away from cytochrome *P*-450 should effectively inhibit any further NADPH-dependent cytochrome *P*-450-mediated oxidation of BHA or BHT. It appears that BHA is required for both of these steps since TBHQ, which forms hydrogen peroxide by itself, is not effective at enhancing BHT toxicity *in vivo* (Thompson and Trush, 1988a). We have also shown that TBHQ itself does not increase the peroxidase-dependent covalent binding of BHT (Thompson and Trush, 1988b).

An interesting observation was that, of the peroxides examined in this study, hydroperoxy-BHT was the most effective peroxide which supported the covalent binding of BHT. Chen and Shaw (1974) first reported hydroperoxy-BHT as a cytochrome *P*-450-mediated metabolite of BHT. This peroxide was subseqeuntly reduced to the corresponding alcohol by the same enzyme and a cyclic pathway was suggested back to the parent compound. Thompson and Wand (1985) also studied the peroxidative metabolism of hydroperoxy-BHT by cytochrome *P*-450. If hydroperoxy-BHT is formed in substantial quantities from the cytochrome *P*-450-mediated oxidation of BHT, perhaps this peroxide is quantitatively more important *in vivo* in driving the peroxidative interaction of BHA

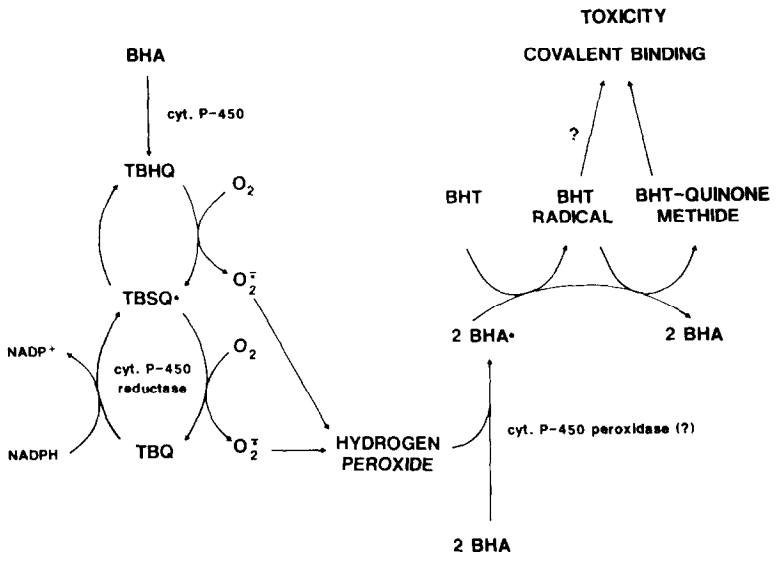

FIG. 3. Possible mechanism for the enhancement of BHT-induced mouse lung toxicity by BHA.

and BHT to form BHT-quinone methide than is hydrogen peroxide from the metabolism of BHA. In any case, either peroxide (hydrogen peroxide or hydroperoxy-BHT) is capable of serving as a substrate for the cytochrome *P*-450 peroxidase reaction suggested in Fig. 3.

Our data suggest the involvement of cytochrome *P*-450 peroxidase in the lung toxicity brought about by the combination of BHA and BHT. Peroxidases have not previously been implicated in BHT-induced toxicity. We have not found classical inhibitors of peroxidase enzymes to be effective in preventing toxicity from high doses of BHT alone. We tested the ability of four such inhibitors—indomethacin, aspirin, methimazole, and propylthiouracil—to inhibit the toxicity elicited by a 400 mg/kg dose of BHT and found that aspirin and methimazole were effective inhibitors but not the other two compounds (data not shown). If typical peroxidase enzymes played a major role in BHT-induced mouse lung toxicity, we would have expected all four compounds to be good inhibitors. We do feel that a peroxidase plays a role in the combination toxicity, however. That cytochrome *P*-450 can reduce various organic hydroperoxides to alcohols and simultaneously oxidize xenobiotic compounds has been known since the early 1970s (Hrycay and O'Brien, 1971) and several types of oxidation reactions have been shown to occur (White and Coon, 1980). However, the significance of the peroxidase activity of cytochrome *P*-450 in the *in vivo* bioactivation of xenobiotics has remained in doubt since the discovery of this activity. Some have questioned whether the conditions necessary for these reactions to be carried out (usually low oxygen, lack of NADPH) actually occur *in vivo*. In our studies, the interaction between the antioxidants occurs under aerobic conditions and NADPH is necessary for the initial oxidation of BHA to TBHQ or BHT to hydroperoxy-BHT. Thus, these two conditions are not limiting. Another problem for implicating cytochrome *P*-450 peroxidase in *in vivo* reactions is the source of the peroxide and the competition for the peroxide by catalase and glutathione peroxidase. In our system the peroxide is generated either at the active site of the cytochrome *P*-450 (in the case of hydroperoxy-BHT) or nearby at the cytochrome *P*-450 (*c*) reductase (in the case of hydrogen peroxide). In either case, it is likely that a significant por-

tion of the peroxide would be available to the cytochrome *P*-450 rather than diffusing away to the peroxisomal catalase or the cytosolic glutathione peroxidase. If correct, our observations would suggest that under certain conditions the peroxidase activity of cytochrome *P*-450 may indeed be involved in the activation of certain xenobiotic compounds *in vivo*.

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