THE PEROXIDATIVE ACTIVATION OF BUTYLATED HYDROXYTOLUENE TO BHT-QUINONE METHIDE AND STILBENEQUINONE

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Butylated hydroxytoluene (BHT, 2,6-di-tert-butyl-4-methyl-phenol) is a commonly used antioxidant allowed in foods in amounts up to 0.02% of the weight of fat present. BHT helps prevent undesirable oxidation reactions from occurring by acting as a free radical scavenger. BHT is also used as a stabilizer in pesticides, gasolines and lubricants, soaps and cosmetics, and as an antiskinning agent in paints and inks (1). BHT has been shown to have a protective effect against the toxicity and carcinogenicity of a wide variety of chemicals (2). However, several recent animal studies have questioned the presumed safety of this antioxidant. For example, BHT has been shown to cause lung damage in mice (3,4), hemorrhagic death in rats (5) and can act as a tumor promoter in both mice and rats (6,7). One of the best characterized toxic effects of BHT is the destruction of type I alveolar and pulmonary endothelial cells (8) in the mouse lung. This lung damage is thought to arise from the biotransformation of BHT into BHT-quinone methide (2,6-di-tert-butyl-4-methylene-2,5-cyclohexadienone) (9,10), a highly reactive compound (see Figure 1). BHT has been demonstrated to be metabolized to BHT-quinone methide in vivo in the mouse (10) and rat (11). This reaction is presumably catalyzed by a cytochrome P-450 related enzyme (12,13). As a class of chemical compounds, quinone methides have been shown to react with cellular nucleophiles including amines, carbohydrates, alcohols, thiols, and olefins (14).

Peroxidase enzymes have recently been shown to catalyze the activation of a wide variety of xenobiotic compounds to reactive intermediates and these enzymes, particularly prostaglandin H synthase, have been suggested to play a role in the extrahepatic toxicity and carcinogenicity of several compounds (15,16). Since antioxidants are good electron donors we investigated whether BHT might be metabolically activated to BHT-quinone methide by peroxidase enzymes. This study reports on the metabolic activation of BHT by two peroxidase enzymes: horseradish peroxidase and prostaglandin H synthase.

METHODS

Materials: (Ring U-^{14}C) BHT (20 mCi/mmole) was purchased from Amersham. Arachidonic acid was obtained from Nu Chek Prep (Elysian, MN). BHT, BHA and other test compounds were obtained from Sigma or Aldrich. Horseradish peroxidase (type II) was obtained from Sigma and prostaglandin H synthase was prepared from ram seminal vesicles obtained from Dr. L. Marnett, Wayne State University. Microsomes were prepared as a 25% homogenate in 0.15 M KCl adjusted to pH 7.8. The homogenate was centrifuged twice at 9,000 g for

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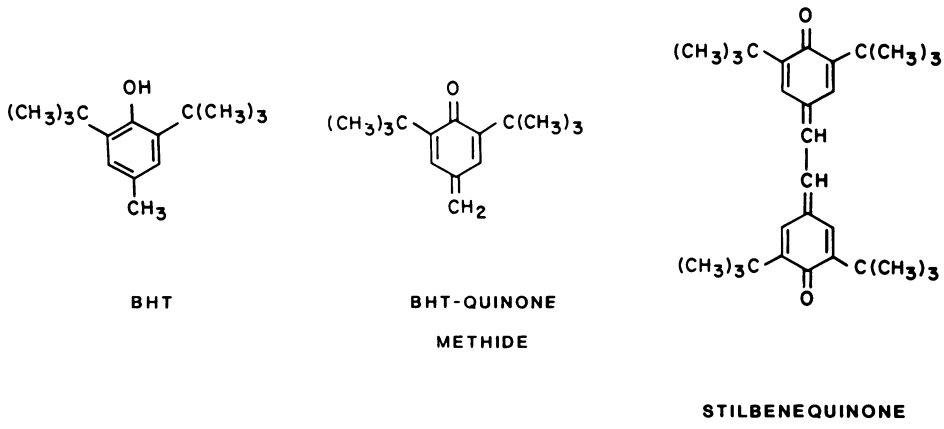

Figure 1. Structures of BHT, BHT-quinone methide and stilbenequinone.

20 minutes. The combined supernatants were filtered through two layers of cheesecloth and then centrifuged at 100,000 g for 1 hour. The final microsomal pellet was resuspended in 0.15 M KCl buffer at a protein concentration of approximately 10 mg/ml and then rapidly frozen in a methanol/dry ice bath and stored at -80°.

Covalent Binding of BHT Metabolite(s) to Microsomal Protein. Reactions were initiated by the addition of arachidonic acid (330 µM) and allowed to proceed for 10 minutes at room temperature. Each tube contained 0.5 µCi BHT (diluted to the appropriate concentration with cold BHT), approximately 1 mg ram seminal vesicle microsomes (specific activity of prostaglandin H synthase 0.52 µmoles arachidonic acid metabolized/minute/mg) in a total of 1 ml of 0.1 M Tris buffer, pH 8.0. BHT and other test compounds were dissolved in DMSO, water, acetone or methanol and added to the reaction in volumes not exceeding 10 µl. Reactions were stopped with 4 ml methanol and the protein pellets were repeatedly extracted with 2 ml methanol or methanol/ether (3:1) until no further radioactivity could be extracted (generally 12-15 washes). Pellets were dissolved in 1 ml of 1 N NaOH and the radioactivity of an aliquot was counted in 10 ml of scintillation fluid. Protein was determined using the Lowry method (17).

Formation of BHT-Quinone Methide and Stilbenequinone. Reactions contained 15 units horseradish peroxidase, 0.9 mM hydrogen peroxide, 200 µM BHT and 100 to 500 µM of the various test compounds in 1 ml of 0.01 M phosphate buffer, pH 7.0. BHT-quinone methide and stilbenequinone (3,5,3',5'-tetra-tert-butylstilbene-4,4'-quinone) were determined to have aqueous absorption maxima of 300 nm and 460 nm respectively and were detected spectrophotometrically. Authentic samples of BHT-quinone methide (18) and stilbenequinone (19) were synthesized and compared to products obtained from these reactions to confirm the identity of these spectral peaks.

RESULTS

We investigated the metabolic activation of BHT to a reactive intermediate(s) by two procedures: (1) assessing the covalent binding of this metabolite to microsomal protein; and (2) monitoring the formation of BHT-quinone methide and its subsequent dimerization product, stilbenequinone (see Figure 1). The concentration dependent covalent binding of BHT to microsomal protein catalyzed by prostaglandin H synthase in the presence of arachidonic acid is presented in Table 1. We have observed that

TABLE 1

Covalent Binding of BHT to Protein Catalyzed by Prostaglandin H Synthase

BHT Concentration	nmoles bound/mg protein[1]
10 μM	0.36 ± .04
50 μM	1.62 ± .19
100 μM	2.84 ± .43
200 μM	4.46 ± .60
500 μM	8.15 ± .78

[1]Values represent mean ± standard error of triplicate determinations.

TABLE 2

Modification of BHT Covalent Binding by Various Compounds[1]

Reaction	% of Control	Type of Radical Formed from Added Compound
Complete system[2]	100[3]	---
+ Glutathione (100μM)	57	---
+ Glutathione (1 mM)	7	---
+ Phenylbutazone	46	Peroxy
+ Diphenylisobenzofuran	84	Peroxy
+ Phenidone	265	N-cation
+ Tetramethylphenylenediamine	223	N-cation
+ BHA	439	Phenoxy
+ Phenol	167	Phenoxy
+ Acetaminophen	151	Phenoxy

[1]Concentration of all test agents was 100 μM except where noted.

[2]Complete system contained 50 μM BHT (0.5 μCi), 1 mg RSV microsomes and 330 μM arachidonic acid in 1 ml 0.01 M phosphate buffer, pH 7.0.

[3]Complete system (100%) = 1.65 ± .09 nmoles BHT bound/mg protein in a 10 minute incubation. Values represent means of triplicate determinations.

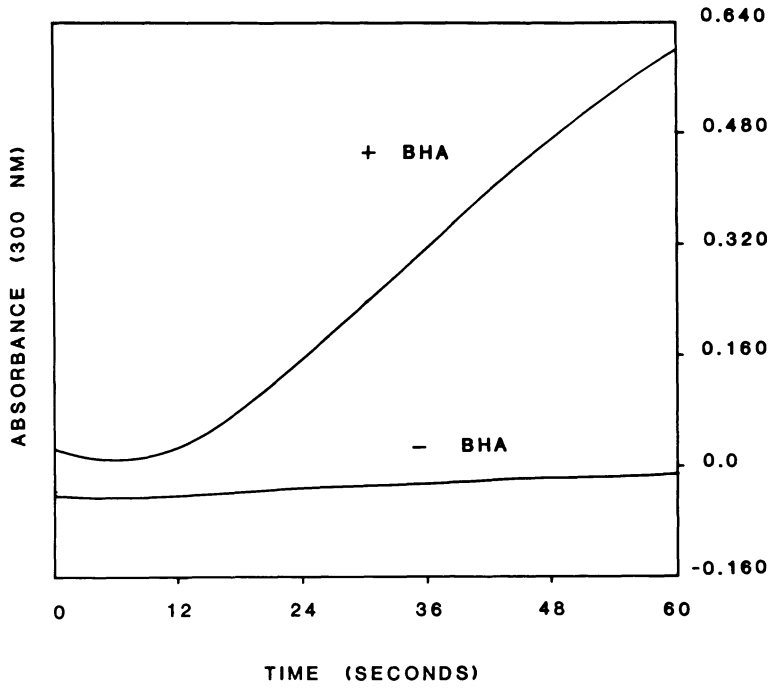

Figure 2. Formation of BHT-quinone methide from horseradish peroxidase-catalyzed oxidation of 200 μM BHT in the presence or absence of 100 μM BHA.

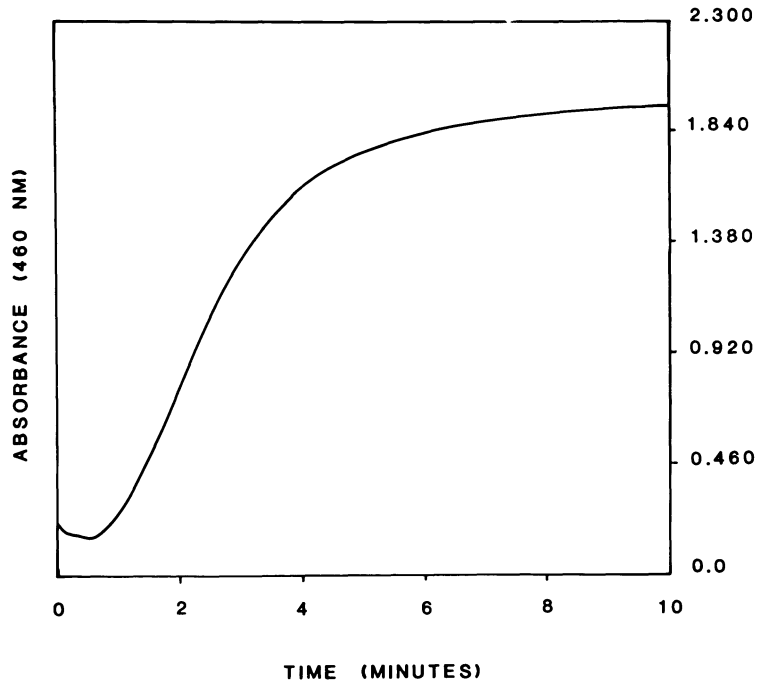

Figure 3. Formation of stilbenequinone from horseradish peroxidase-catalyzed oxidation of 200 μM BHT in the presence of 100 μM BHA.

hydrogen peroxide can also stimulate BHT covalent binding in this system indicating the peroxidative nature of this activation reaction (not shown). The ability of various pharmacologic agents to modify the covalent binding of BHT is shown in Table 2. Glutathione was an effective inhibitor of BHT binding. In the presence of butylated hydroxyanisole (BHA), however, the covalent binding of BHT was greatly enhanced and BHT-quinone methide was detected as a metabolic intermediate (see Figure 2). Several other compounds which are known to be cooxidation substrates for prostaglandin H synthase were also tested for their ability to influence the covalent binding of BHT. These included phenylbutazone and diphenylisobenzofuran which form peroxy radicals, phenidone and tetramethylphenylenediamine which form nitrogen-centered cation radicals, and phenol and acetaminophen which form phenoxy radicals when activated by peroxidase enzymes. The results in Table 2 demonstrate that several of these compounds stimulated BHT metabolism and that this stimulation may be related to the type of radical intermediate formed by the compound.

The formation of BHT-quinone methide from the horseradish peroxidase-catalyzed oxidation of BHT is shown in Figure 2. A small amount of BHT-quinone methide (1.18 nmoles/minute using an extinction coefficient of 27,000 $M^{-1}cm^{-1}$ from reference 18) was detected during the metabolism of 200 μM BHT in the absence of any activators. In the presence of 100 μM BHA the formation of BHT-quinone methide was greatly enhanced (26.6 nmoles/minute). The increased formation of this metabolite may thus be responsible for the increased covalent binding of BHT that was seen in the presence of BHA (see Table 2). In the presence of BHA, we also observed the formation of stilbenequinone. The formation of stilbenequinone was not observed from BHT alone, even at 1 mM BHT. The formation of stilbenequinone was preceeded by a lag period of about 1 minute after the start of the reaction (see Figure 3) supporting the contention that BHT-quinone methide was formed initially and subsequently dimerized to form the stilbenequinone. In addition to the compounds reported here, we have also observed that several other compounds can enhance the covalent binding of BHT. These include diethylstilbestrol, estradiol, methimazole, guaiacol, methyl paraben and eugenol. Compounds which enhanced BHT binding were also observed to enhance the formation of BHT-quinone methide and stilbenequinone whereas compounds which inhibited BHT binding did not enhance BHT-quinone methide or stilbenequinone formation (not shown).

The effect of glutathione on the horseradish peroxidase-catalyzed formation of BHT-quinone methide from 200 μM BHT in the presence of 100 μM BHA is shown in Figure 4. 100 μM glutathione inhibited BHT-quinone methide formation by approximately 50% while 500 μM glutathione completely inhibited the formation of BHT-quinone methide. The formation of stilbenequinone was similarly inhibited. If 100 μM glutathione was added to the reaction mixture after BHT-quinone methide had already been formed, the peak representing BHT-quinone methide rapidly disappeared suggesting the possible formation of a BHT-glutathione conjugate. During such reactions glutathione may also be oxidized to GSSG with the concommitent conversion of the BHT metabolite back to BHT.

DISCUSSION

This report demonstrates that the peroxidase-mediated activation of BHT results in the formation of a reactive intermediate(s) capable of covalently binding to microsomal protein. Since only a small amount of BHT-quinone methide was detected spectrophotometrically from the metabolism of BHT alone, perhaps BHT-quinone methide is not the only BHT metabolite responsible for this binding, as has been suggested in the literature (13). Peroxidase enzymes utilize one electron donors as cofactors and thus BHT should form a radical (a one electron oxidation product) prior to forming BHT-quinone

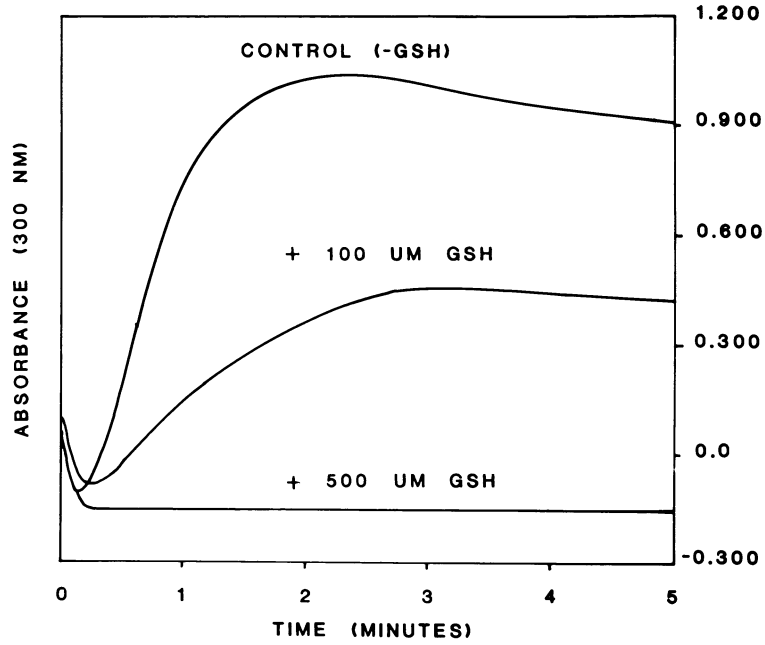

Figure 4. Effect of glutathione (GSH) on the formation of BHT-quinone methide from horseradish peroxidase-catalyzed oxidation of 200 μM BHT in the presence of 100 μM BHA.

methide (a two electron oxidation product). This BHT radical might be responsible for the covalent binding of BHT in the absence of any activators. In the presence of activators such as BHA, the increase in the formation of BHT-quinone methide may account for the increase in covalent binding. Thus, both a free radical of BHT and BHT-quinone methide may be involved in the covalent binding of BHT to tissue macromolecules and hence its toxicity.

In addition to BHA, several other compounds were capable of enhancing BHT binding and BHT-quinone methide formation. It appears that the ability of a compound to enhance BHT binding depends on whether it can form a radical, what type of radical intermediate it forms, and whether BHT interacts with the radical by donating an electron to it. A possible mechanism for the enhancement of BHT binding by these compounds is suggested in Figure 5. As illustrated in the schematic, a xenobiotic compound (XH) is preferentially oxidized by the peroxidase to a radical ($X^{\cdot}$). In the presence of BHT, this xenobiotic-derived radical would be recycled back to the parent molecule by accepting an electron from BHT. The end result is the regeneration of the parent xenobiotic molecule and the enhanced conversion of BHT to a radical first, then BHT-quinone methide and ultimately stilbenequinone. A similar mechanism has been proposed by Kurechi and Kato (20) for a non-enzymatic system utilizing tert-butyl hydroperoxide as the oxidizing agent. In their system, BHA enhanced the metabolism of BHT to BHT-quinone methide. As a consequence, the parent BHA molecule was regenerated and thus accounted for an increased antioxidant capacity observed in the presence of both BHT and BHA.

Most chemicals which have been approved for use in food and cosmetic products have been tested for their toxicological effects on an individual basis. Toxic effects resulting from the interaction of two or more compounds are more difficult to predict or assess, yet nevertheless may be very important. Recently Reed et al. (21) observed that phenylbutazone enhanced the prostaglandin H synthase catalyzed epoxidation of 7,8-dihydroxy-7,8-

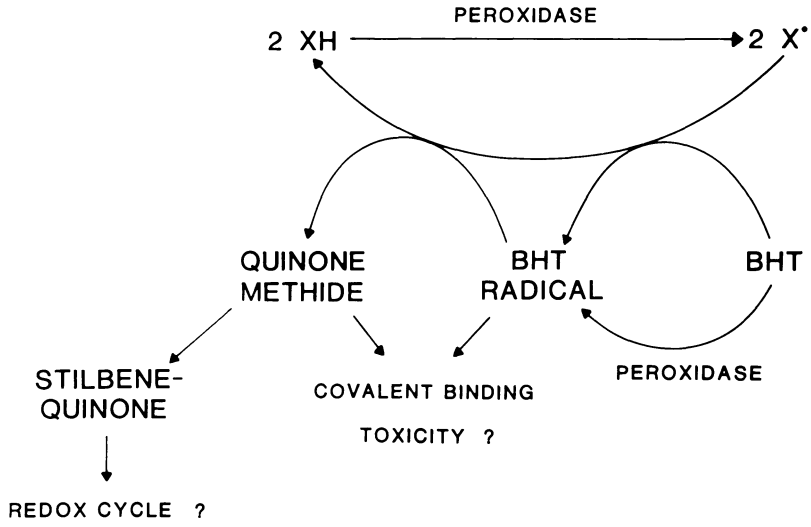

Figure 5. Possible mechanism for the formation of BHT-quinone methide and stilbenequinone from the peroxidase-catalyzed oxidation of BHT in the presence of a xenobiotic activator (XH).

dihydrobenzo[a]pyrene. The primary event was the formation of a peroxy radical from phenylbutazone which appeared to be the epoxidizing agent. The same type of chemical-chemical interaction is proposed here with the end result being the enhanced formation of the toxic BHT-quinone methide.

The formation of stilbenequinone may also be of toxicologic significance since this compound may be a substrate for cellular enzymes such as cytochrome P-450 reductase and thus redox cycle and generate reactive oxygen species. These reactive oxygen species may further contribute to BHT-induced toxicity.

We have clearly demonstrated that peroxidase enzymes are capable of metabolizing BHT to BHT-quinone methide in the presence of several activator compounds. This observation suggests that the involvement of peroxidase enzymes in the mechanism of toxicity of BHT should be examined. Since there are apparently a number of compounds which can serve as activators of BHT metabolism in vitro, perhaps there are endogenous compounds which can serve as activators in vivo as well. There are many phenolic compounds naturally present in foods (and thus ingested along with BHT) as well as many endogenous phenols which might serve as such an activator. The presence of an endogenous activator in a tissue with substantial peroxidase activity might render that tissue susceptible to BHT toxicity.

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