2(3)-Tert-Butyl-4-Hydroxyanisole Inhibits Oxidative Metabolism of Aflatoxin B_1 in Isolated Rat Hepatocytes (42952)

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Abstract. **Previous studies indicate that dietary administration of phenolic antioxidants, 2(3)-tert-butyl-4-hydroxyanisole (BHA) and 3,5-di-tert-butyl-4-hydroxytoluene, inhibits the carcinogenic effect of a number of chemical carcinogens including aflatoxin B_1 (AFB_1). Induction of hepatic enzymes, such as glutathione *S*-transferase, UDP-glucuronyltransferase, and epoxide hydrolase, has been shown to be responsible for the reduction of AFB_1 cytotoxic and carcinogenic effects. The effect of BHA on AFB_1 activation was examined *in vitro* utilizing isolated rat hepatocytes and liver microsomes. In hepatocytes, the total AFB_1 content and bound form of AFB_1 were 3.4 and 1.4 pmol/10^6 cells, respectively. In the cell-free microsomal activating system, 2.2 pmol were activated per mg of microsomal protein during 60 min of incubation. BHA (0.1–0.5 m*M*) inhibited AFB_1 activation and binding in both systems in a dose-dependent manner; in hepatocytes, 90% inhibition was observed at 0.5 m*M*. Analyzing various AFB_1 adducts, BHA (0.25 m*M*)-treated hepatocytes contained a significantly reduced amount of AFB_1 macromolecular adducts. The antioxidant neither stimulated nor inhibited the cytosolic glutathione *S*-transferase and microsomal UDP-glucuronyltransferase activities. Analysis of various hydroxylated (aflatoxins M_1 and Q_1 (AFM_1 and AFQ_1)) and demethylated (aflatoxin P_1 (AFP_1)) metabolites of AFB_1 in both the conjugated and unconjugated form indicated that there was a 30–50% reduction of unconjugated AFP_1, AFQ_1, and AFM_1, whereas AFB_1 was increased 3-fold. There was no significant change of conjugated metabolites. The effect of BHA on AFB_1 activation in hepatocytes was compared with that of other cytochrome P-450 inhibitors; the ED_{50} values of SKF 525A, BHA, and metyrapone were 9 μM, 40 μM, and 280 μM, respectively. In the cell-free microsomal system, biotransformation of AFB_1 to AFP_1, AFM_1, and AFQ_1 was also inhibited. Kinetic analysis of *p*-nitroanisole *O*-demethylase activity of rat liver microsomes demonstrated that BHA inhibited noncompetitively with an apparent K_i of 90 μM. In the absence of enzyme induction, the phenolic antioxidant, BHA, blocks the oxidative biotransformation of AFB_1 in isolated hepatocytes.** [P.S.E.B.M. 1989, Vol 192]

It has been known for many years that addition of some vitamins, reduced glutathione, phenolic antioxidants, and other naturally occurring substances, to human and animal food may lower the incidence of cancer caused by chemical carcinogens. The mechanisms of action of most of these agents are not fully understood (1, 2). Wattenberg (1) divided them into three categories: compounds preventing the formation of carcinogen from precursor compounds, compounds preventing the carcinogen from reaching and reacting with the target site(s), and compounds suppressing the expression of neoplasia. Dietary administration of phenolic antioxidants such as 2(3)-tert-butyl-4-hydroxyanisole (BHA) and 3,5-di-tert-butyl-4-hydroxytoluene (BHT) is known to inhibit the carcinogenic effect of benzo(*a*)pyrene (3), 7,12-dimethylbenz(*a*)anthracene (4), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (5), and aflatoxin B_1 (AFB_1) (6) in tissues of rodents. It is generally accepted that phenols act at multistages in modulating carcinogenesis (1, 2). Administration of BA to male Sprague-Dawley rats increased the specific activities of liver glutathione *S*-transferase (7) and epoxide hydrolase (8). In female CD-1 mice, however, these enzyme activities were elevated in both liver and extrahepatic tissues (9). In rat liver micro-

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somes, the epoxide hydrolase was 2.8-fold greater in BHA-treated animals in comparison to controls, whereas the glutathione *S*-transferase activity was increased by 1.8-, 2.0-, and 2.1-fold with *p*-nitrobenzyl chloride, 1-chloro-2,4-dinitrobenzene (CDNB), and 1,2-dichloro-4-nitrobenzene, respectively, as substrates (7, 8). In mice, marked increases were seen in liver epoxide hydrolase (11.1-fold) and glutathione *S*-transferase (8–11-fold), with smaller increases in extrahepatic tissues (9). Therefore, Benson *et al.* (9) suggested that the observed anticarcinogenic effects of BHA and other phenolic antioxidants might be due to induction of enzymes involved in the removal of reactive metabolites. AFB_1, a hepatotoxic, mutagenic, and carcinogenic mycotoxin present in human food and animal feed, is converted to AFB_1-8,9-epoxide and a number of polar metabolites such as aflatoxin P_1 (AFP_1), aflatoxin M_1 (AFM_1), and aflatoxin Q_1 (AFQ_1) by liver cytochrome P-450 isozymes (10–13). The activated epoxide forms covalent adducts with DNA, RNA, and proteins (14–17). The reactive epoxide may be removed by conjugating with reduced glutathione (GSH) catalyzed by glutathione *S*-transferase and by the action of epoxide hydrolase to form AFB_1-dihydrodiol. The various polar metabolites can be eliminated by the formation of water-soluble conjugates with sulfate or glucuronic acids (18–21). Several whole animal studies reveal dietary BHA as effectively reducing the *in vivo* AFB_1-DNA binding that is accompanied by enhanced activity of glutathione *S*-transferase and UDP-glucuronyltransferase, and epoxide hydrolase (22–25). These studies substantiate the findings of Benson and coworkers (7–9). Therefore, the induction of detoxication enzymes by dietary BHA has been considered to play a central role in the long-term *in vivo* prevention of cancer (26, 27). In the search for factors modulating AFB_1 binding to DNA *in vitro*, Bhattacharya and coworkers reported that BHA and many other compounds inhibited microsome-mediated binding of AFB_1 to added calf thymus DNA in a cell-free system (28) and the formation of AFB_1-8,9-epoxide (29). These findings suggest that the mechanism of action of BHA and other phenolic antioxidants may not only involve the induction of detoxifying enzymes but may also cause alterations of the microsomal monooxygenase system. To examine the initial and direct effect of BHA on oxidative metabolism of AFB_1 without the influence of dietary induction of detoxifying enzymes, we have employed the isolated rat hepatocyte system to study the formation of AFB_1 adducts and polar metabolites in the presence and absence of added BHA.

Materials and Methods

Chemicals. All chemicals were of the highest purity available. Phenol (redistilled nucleic acid grade) was from Bethesda Research Laboratories, Bethesda, MD. Aflatoxins (AFB_1, AFP_1, AFM_1, and AFQ_1), BHA (90% 3-tert isomer and 10% 2-tert isomer), BHT, α-tocopherol acetate (TPA), GSH, 1,2-epoxy-3,3,3-trichloropropane (TCPO), CDNB, *p*-nitroanisole, 4-nitrophenol, UDP-glucuronic acid, β-glucuronidase, sulfatase, glucose-6-phosphate dehydrogenase, glucose 6-phosphate, $NADP^+$, RNase A, yeast RNA, calf thymus DNA, proteinase K, metyrapone, ultra-pure sucrose, and bovine serum albumin were from the Sigma Chemical Co., St. Louis, MO. [^{3}H]AFB_1 (15–30 Ci/mmol) was obtained from Moravek Biochemical, Brea, CA. OCS, ACS II scintillants, and NCS tissue solubilizer were from Amersham Corp., Arlington Heights, IL. SKF 525A was a gift of the Smith Kline & French Laboratories, Philadelphia, PA. Silica gel plates and spectro grade solvents were obtained from Fisher Scientific Co. (Pittsburgh, PA). Other chemicals were purchased from sources as previously described (30).

Animals. Male Wistar rats (250–300 g) were supplied by Harlan Co., Indianapolis, IN. They were maintained on a 12-hr light/dark cycle and allowed water and Purina Chow *ad libitum*. Hepatocytes were prepared according to the procedure of Berry and Friend (31) with modifications as described previously (30). In experiments using livers, animals were anesthetized with Ketaset (16 mg/100 g body wt, and livers were perfused *in situ* with 0.1 *M* sodium phosphate buffer (pH 7.4), excised, and rinsed in perfusing buffer containing 0.25 *M* sucrose (SSP buffer).

Incubations. In experiments employing intact hepatocytes or isolated microsomes, conditions were similar to those in reports published previously from this laboratory (30, 32). In the present study, hepatocytes (25×10^6 cells) were incubated in a final volume of 50 ml. Cells or microsomes were preincubated with various agents for 15 min before the addition of [^{3}H]AFB_1. After incubation, aliquots of the cell suspension were removed for determination of cell numbers, cell viability, and cellular content (total and bound) of [^{3}H]AFB_1 as described (30). The remaining suspension was used to isolate various subcellular fractions, to determine AFB_1-DNA and -RNA adducts, and to study the oxidative metabolism of AFB_1.

Isolation of Microsomal and Cytosolic Fractions. In experiments employing intact hepatoctyes, cells were washed, packed, and resuspended in 5.0 ml of isolation buffer containing 0.25 *M* sucrose/1 m*M* EDTA/2 m*M* Tricine (pH 7.6) and sonicated for 45 sec at 45 W in three 15-sec bursts at 4°C in a Sonifier cell disrupter (Heat-system, model W185). The volume of the cell lysate was then brought to 10 ml and centrifuged for 10 min at 400*g*; then the supernatant was centrifuged for 10 min at 10,000*g* and again for 60 min at 105,000*g* The pellet was taken as microsomes and the supernatant was taken as cytosolic fraction (33). In experiments employing intact liver, livers were homogenized in 6 vol of SSP buffer with six strokes of a motor-driven Teflon-glass Potter-Elvehjem homogenizer. The ho-

mogenate was filtered through four layers of cheesecloth and centrifuged twice at 9770*g* for 15 min at 4°C, with discarding of the pellets each time. The resulting supernatant fraction was centrifuged for 60 min at 105,000*g*; the microsomal pellet was resuspended in SSP buffer and the supernatant was taken as cytosolic fraction.

Estimation of AFB_1 Binding to DNA and RNA. After incubation, hepatocytes (25×10^6 cells) were packed, washed, and resuspended in fresh incubation buffer. Total nucleic acid fraction containing both DNA and RNA was extracted with phenol-chloroform, precipitated by ethanol, and dissolved in 0.1 *M* sodium acetate (pH 5.0); then, radioactivity was determined. DNA was isolated according to the method described by Mathew (34) after incubation with RNase A and proteinase K to remove RNA and protein. The radioactivity of [^{3}H]AFB_1 present in the DNA was determined. The difference of [^{3}H]AFB_1 radioactivity between total nucleic acid fraction and DNA was taken as [^{3}H]AFB_1 bound to RNA.

Microsome-mediated Activation of AFB_1. Liver microsomes (5.0 mg) were incubated for 5 min with various agents before the addition of [^{3}H]AFB_1. The incubation mixture contained an NADPH-generating system (glucose 6-phosphate, 3.4 m*M*; glucose-6-phosphate dehydrogenase, 10 units; $NADP^+$, 0.5 m*M*) in a final volume of 5.0 ml of 0.1 *M* sodium phosphate buffer (pH 7.4). After 60 min, 50 μl of incubation mixture were removed and added to 10 ml of 10% trichloroacetic acid. The acid-insoluble radioactivity was determined by liquid scintillation spectrometry (33). The remaining microsomal incubation mixture was extracted with chloroform as described below to study the oxidative metabolism of AFB_1.

Isolation, Separation, and Identification of Various Oxidative Metabolites of AFB_1. Hepatocytes (50×10^6 cells) were washed twice, packed, and resuspended in 4.0 ml of isolation buffer (pH 7.6). The cell suspension was extracted twice, each time using 6.4 ml of chloroform. The chloroform extracts were quantitatively removed and evaporated to dryness. This extract is called CE-1 and contains unconjugated AFB_1 and its metabolites. The aqueous fraction was adjusted to pH 5.0 with 0.4 *M* sodium acetate/acetic acid buffer, similar to the procedure described by Wei *et al.* (35). The mixture was centrifuged at 17,400*g* for 15 min to remove any precipitates. The supernatant was incubated at 37°C overnight (16–18 hr) with β-glucuronidase (48,000 units) and sulfatase (1,700 units). The incubation mixture was again centrifuged and the supernatant was extracted with chloroform as described above. This extract is called CE-II and contains AFB_1 metabolites derived from their glucuronide and sulfate conjugates. The aqueous fraction was then acidified with HCl to 0.2 *N* and heated in a boiling water bath for 2 hr. The acid hydrolysate was extracted with chloroform as described above. This extract is called CE-III and represents mercapturate conjugates. The remaining aqueous phase contains AFB_1-GSH conjugate (35). The chloroform extracts were quantitatively removed and dried, and the residues were dissolved in 100 μl of methanol. The metabolites were separated by thin-layer chromatography (TLC) on precoated silica gel plates (20 × 20 cm, Kodak) using chloroform/methanol (95:5) as the solvent system (36). Each lane of the plate was cut into 0.5-cm slices and each slice was placed into a counting vial. The radioactivity was determined by liquid scintillation spectrometry. The various aflatoxins were identified by their R_f values with authentic standards. The R_f values for AFP_1, AFM_1, AFQ_1, and AFB_1 were 0.26, 0.55, 0.78, and 0.87, respectively. Using [^{3}H]AFB_1 standard, the recovery of radioactivity after chloroform extraction, reconstitution and TLC separation was 42 ± 13%, the mean ± SD of nine experiments. Radioactivities were presented as 10^4 dpm/10^6 cells without correction for nonrecoverable radioactivity.

Determination of Enzyme Activities. Glutathione *S*-transferase activity of the cytosolic fraction isolated from hepatocytes incubated with and without BHA was determined with CDNB and glutathione as substrates. The initial rates of the thioether formation were measured at 340 nm in a Gilford recording spectrophotometer at 25°C, according to the procedure described by Habig and Jakoby (37). Microsomal UDP-glucuronyltransferase activity was determined using 4-nitrophenol and UDP-glucuronate as substrates, as described by Burchell and Weatherill (38). *p*-Nitroanisole *O*-demethylase activity present in the microsomal fraction was determined according to a modification of the method of Kato and Gillette (39), which measures the conversion of *p*-nitroanisole to *p*-nitrophenol. The molar extinction coefficient is 18.75×10^3 for nitrophenol at 404 nm.

Analytical Methods. Cell concentrations and viability were determined in an American Optical Neubauer Hemacytometer as described previously (30). Protein was assayed by the method of Lowry *et al.* (40). DNA and RNA were determined as described previously (41).

Results

Effect of BHA on Intracellular Content and Bound AFB_1. The influence of BHA and various compounds on the cellular content and bound form of AFB_1 is shown in Table I. Hepatocytes (0.5×10^6 cells/ml) were incubated with various agents for 15 min before the addition of [^{3}H]AFB_1 to a final concentration of 50 n*M*, and the incubation was continued for another 60 min. The untreated cells contained 3.4 ± 0.5 pmol/10^6 cells of AFB_1, and 1.4 ± 0.2 pmol/10^6 cells of AFB_1 were present as the bound form. At the concentrations tested, KCN, TPA, and BHT had no significant effect. The lack of inhibition by TPA was similar to the lack of effect with the microsome-mediated binding of [^{3}H]

AFB_1 to added calf thymus DNA (28). KCN at 2 m*M* showed no effect on AFB_1 content and binding, suggesting that AFB_1 uptake and binding to macromolecules were not energy dependent. The effects of TCPO, GSH, metyrapone, and SKF 525A on the AFB_1 forms were similar to our previous findings (30, 41), indicating that hepatocytes isolated from untreated animals were capable of metabolizing AFB_1. The lack of effect by BHT on the total AFB_1 content and bound form indicates that metabolism of BHA and BHT in isolated hepatocytes is different. In microsome-mediated activation of AFB_1 *in vitro*, Bhattacharya and co-workers reported that BHT, at 0.1 m*M*, decreased the formation of the AFB_1-DNA adduct (28) and AFB_1-8,9-epoxide (29) to 27.8% and 26.2% of the control, respectively. In rats receiving BHT (0.5%) in the diet, Cha and Heine (42) observed elevations of cytochrome P-450 (2.1-fold), NADPH-cytochrome *c* reductase (1.5-fold), epoxide hydrolase (5.3-fold), UDP-glucuronyltransferase (2.5-fold), and GSH-transferase (2.8-fold). A closer examination of the effect of phenolic antioxidants on AFB_1 activation in a cell-free system (28, 29), in intact animals (22–25), and in isolated hepatocytes (Table I) reveals that different cellular processes are involved.

Effect of Various Concentrations of BHA on AFB_1 Activation. It is known that oxidative metabolism of AFB_1 is catalyzed primarily by microsomal cytochrome P-450 isozymes (13) and, to a lesser extent, by the nuclear (36) and mitochondrial (43) enzyme systems. The formation of covalently bound AFB_1 to macromolecules takes place at various cellular sites, and the extent of modification of cellular components may vary depending on the concentration of BHA. As shown in Figure 1, the cellular content and total bound form of AFB_1 decreased with increasing concentration of BHA, and at 0.5 m*M* BHA, 90% inhibition was observed. Examination of the formation of various AFB_1 adducts (Fig. 1, inset) showed that BHA, at 0.25 m*M*, inhibited the formation of DNA and RNA adducts by 82% and 79%, respectively. When the formation of AFB_1-protein adduct was calculated, a similar inhibition was also noted. These data suggest that BHA may prevent either the formation of the AFB_1-epoxide extranuclearly or its nuclear translocation or both. Based on the total bound form of AFB_1 (Fig. 2), the ED_{50} of BHA to inhibit AFB_1 activation in isolated hepatocytes was estimated to be 40 μM. Similar studies were conducted with SKF 525A and metyrapone, inhibitors of cytochrome P-450 enzymes; the ED_{50} values for SKF 525A and metyrapone were 9 μM and 280 μM, respectively. BHA produced a stronger inhibition than metyrapone and weaker inhibition than SKF 525A on AFB_1 activation. BHA behaved analogously to both SKF 525A and metyrapone, suggesting that BHA prevents the formation of AFB_1-8,9-epoxide by modulating the liver cytochrome P-450 enzymes during the incubation of hepatocytes.

Effect of BHA on Microsomal Activities. Employing microsomes isolated from untreated rats, the *in vitro* microsome-mediated AFB_1 activation measured as acid-insoluble radioactivity was significantly inhibited (27.3% of the control) by 0.25 m*M* BHA (Table II). This finding is similar to that of Bhattacharya *et al.* (28), who reported that binding of AFB_1 to added DNA was 16% and 2% of the control with BHA at 0.1 and 0.5 m*M*, respectively. The cell-free, microsome-me-

Table I. Factors Affecting AFB_1 Content and Binding in Isolated Hepatocytes

Incubation condition	Relative changes of intracellular AFB_1	
	Content (%)	Bound (%)
Complete	100[a]	100[a]
+TCPO (0.5 m*M*)	230[b]	300[b]
+KCN (2.0 m*M*)	92	104
+TPA (1.0 m*M*)	85	86
+BHT (0.25 m*M*)	79	86
+GSH (0.25 m*M*)	73	68
+BHA (0.25 m*M*)	35[b]	21[b]
+Metyrapone (1.0 m*M*)	20[b]	28[b]
+SKF 525A (50 μM)	9[b]	9[b]

[a] The 100% values represents 3.4 ± 0.5 and 1.4 ± 0.2 pmol/10^6 cells of the total and bound forms of AFB_1, the mean ± SD, from four experiments, respectively.
[b] $P < 0.05$.

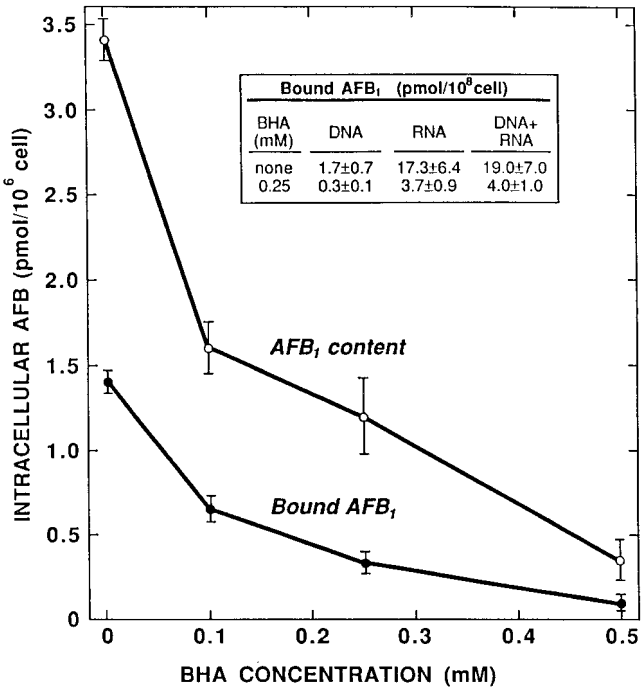

Figure 1. Effect of BHA on AFB_1 content and binding to macromolecules in isolated hepatocytes. Incubation conditions were similar to those in Table I. At each BHA concentration, AFB_1 content (○) and bound AFB_1 (●) represent the average of at least five experiments, and the bar indicates the SE. In the inset, data presented are mean ± SD from four experiments.

diated AFB_1 activation was also inhibited by metyrapone. In addition, the microsomal *p*-nitroanisole *O*-demethylase activity was clearly inhibited greater than 60% by BHA (Table II). As depicted by the Dixon plot in Figure 3, BHA is a noncompetitive inhibitor of *p*-nitroanisole-*O*-demethylease, and the K_i was estimated to be 90 μM. The data obtained from intact hepatocytes (Fig. 1) and isolated liver microsomes (Table II and Fig. 3) suggest that either BHA or its metabolite interacts with the microsomes and causes changes in the microsomal monoxygenase activity.

Effect of BHA on Other Oxidative Metabolites of AFB_1. The hydroxylated (AFM_1 and AFQ_1) and demethylated (AFP_1) derivatives are the products of oxidative biotransformation of AFB_1 by the cytochrome P-450 enzymes. These metabolites are released from the cell as such or are conjugated as glucuronides, sulfates, or glutathione conjugates. The conjugates can be hydrolyzed by digestion with enzymes or 2 *N* HCl and extracted with chloroform (35). The remaining aqueous phase contains, among others, the AFB_1-GSH conjugate (44). As depicted in Table III, CE-I showed a significant increase of [^{3}H]AFB_1 radioactivity (2.4-fold) in BHA-treated cells, whereas the water-soluble fraction contained only 25% of the radioactivity present in the untreated cells. CE-II and CE-III fractions exhibited no significant differences between untreated and

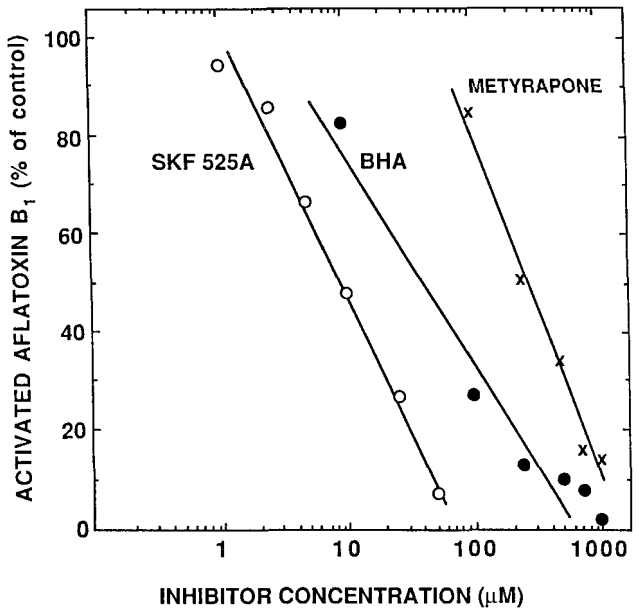

Figure 2. Concentration-dependent inhibition of AFB_1 activation by SKF 525A, BHA, and metyrapone in isolated hepatocytes. Incubation conditions were similar to those in Table I. Data presented are the average of two to three experiments. O, SKF 525A; ●, BHA; X, metyrapone.

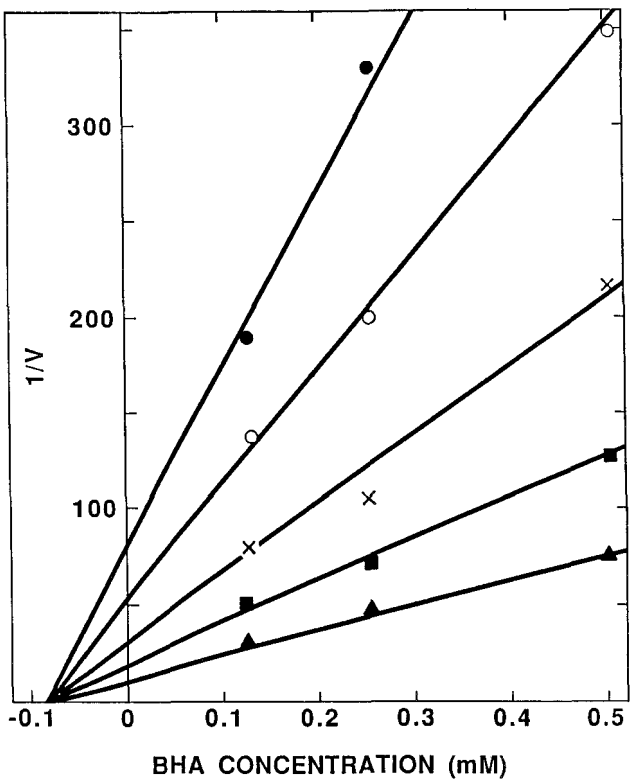

Figure 3. Dixon plot for *p*-nitroanisole *O*-demethylase activity at five substrate concentrations in the presence of increasing amounts of BHA. Each value represents the mean of four experiments from microsomes pooled from 12 rats. Concentrations of substrate (m*M*) ●, 0.26; O, 0.51; X, 1.53; ■, 3.06; and ▲, 6.13. V, initial reaction velocity expressed as nmol of *p*-nitrophenol formed/min/mg of microsomal protein.

Table II. Effect of BHA and Metyrapone on AFB_1 Activation and *O*-Demethylase Activity in Isolated Liver Microsomes[a]

Microsomal incubation system	AFB_1 Activation[b] (pmol/mg protein)	*p*-Nitroanisole[c] *O*-Demethylase (pmol/min/mg)
+None	2.2 ± 0.2 (4)	45.4 ± 5.7 (6)
+BHA (0.25 m*M*)	0.6 ± 0.1 (4)*	15.3 ± 7.7 (5)*
+Metyrapone (1.0 m*M*)	0.4 ± 0.1 (4)*	29.1 ± 5.6 (3)*

[a] Data presented are mean ± SD from number of experiments given in parentheses; * indicates $P < 0.05$.
[b] Final concentration of [^{3}H]AFB_1 was 3.3 n*M* and the mixture was incubated for 60 min under 95% O_2-5% CO_2.
[c] The microsomes were pretreated with or without BHA or metyrapone for 15 min at room temperature before the addition of the substrate, *p*-nitroanisole. The mixture was incubated for 40 min at 37°C under 95% O_2-5% CO_2.

BHA-treated cells. These results indicate that the formation of various conjugates in the liver cells was not enhanced by the antioxidant. To investigate further the unconjugated metabolites, CE-I fraction was concentrated and metabolites were separated by silica gel TLC. As shown in Table IV, BHA reduced the relative proportion of unconjugated AFP_1, AFM_1, and AFQ_1 with an increase in AFB_1. The radioactivity was expressed as 10^4 dpm/10^6 cells; control values were 0.10, 0.06, 0.02, and 0.37, and in the BHA-treated cells they were 0.07, 0.04, 0.01, and 1.10, respectively, indicating that, while the biotransformation of AFB_1 to AFP_1, AFM_1, and AFQ was inhibited by 30–50%, the unmetabolized AFB_1 was accumulated almost 3-fold. This accumulation of AFB_1 could be accounted for by the 76% decrease of AFB_1-epoxide. When the epoxide was determined as bound AFB_1 by trichloroacetic acid-insoluble radioactivity (Fig. 1), the untreated cell contained 1.40 pmol/10^6 cells of the bound form of AFB_1, whereas the BHA (0.25 m*M*)-treated cells contained 0.34 pmol/10^6 cell. The net decrease of AFB_1-epoxide is 1.0^6 pmol, and it represents 48% of the decrease of AFB_1 content elicited by BHA treatment. These results further suggest that in intact hepatocytes, BHA seems to exert its inhibitory effect on the cytochrome P-450 enzymes; thus, it prevents the oxidative biotransformation of AFB_1 rather than the removal of reactive and toxic polar metabolites.

Table III. Influence of BHA on the Formation of Unconjugated and Conjugated Metabolites of AFB_1 in Isolated Hepatocytes[a]

Chloroform extracts[b]	Radioactivity (dpm × 10^4/10^6 cells)	
	Untreated hepatocyte	BHA-treated hepatocyte
CE-I	0.55 ± 0.30 (21)	1.30 ± 0.60 (11)*
CE-II	0.17 ± 0.08 (11)	0.13 ± 0.06 (4)
CE-III	0.33 ± 0.11 (8)	0.13 ± 0.08 (4)
H_2O-phase	2.5 ± 0.80 (13)	0.61 ± 0.37 (4)*

[a] Data presented are mean ± SD from number of experiments given in parentheses; * indicates $P < 0.05$. Incubation conditions were similar to those in Table I.
[b] CE-I, chloroform extract before enzyme digestion, contains AFB_1 and unconjugated metabolites; CE-II, extract after enzyme digestion, contains glucuronide and sulfate conjugates; CE-III, extract after acid hydrolysis, contains mercapturate. Remaining H_2O-phase contains AFB_1-GSH conjugates (44).

Effect of BHA on Microsomal and Cytosolic Enzyme Activities. It is important to note that, since hepatocytes were isolated from untreated adult male rats, the effect of BHA on the activity of preexisting enzymes can be easily studied in the isolated hepatocytes system without the influence of dietary induction of enzymes, as is frequently observed in whole animal studies (1, 2). As shown in Table V, BHA-treated hepatocytes did not show any changes of GSH-transferase or UDP-glucuronyltransferase activity in the cytosolic and microsomal fractions after the cells were incubated with the antioxidant for 60 min at 37°C under 95% O_2 and 5% CO_2.

Discussion

We have described the effect of BHA on AFB_1 metabolism and its binding to macromolecules in isolated hepatocyte of normal rat. This study demonstrated that in the absence of induction of detoxifying enzymes such as glutathione *S*-transferase and UDP-glucuronyltransferase, BHA reduced the formation of the reactive AFB_1-epoxide. Consequently, various macromolecular AFB_1 adducts were greatly decreased. Sev-

Table IV. Relative Level of Polar Oxidative Metabolites of AFB_1 in Untreated and BHA-Treated Hepatocytes[a]

Treatment	Radioactivity (% of CE-I)[b]				
	AFB_1	AFP_1	AFM_1	AFQ_1	Unidentified
Untreated hepatocytes	66.7 ± 6.9 (17)	18.1 ± 4.7 (16)	10.1 ± 2.9 (16)	4.4 ± 2.7 (16)	7.5 ± 3.9 (9)
BHA-treated hepatocytes	85.5 ± 8.6* (10)	5.4 ± 3.5* (11)	3.4 ± 0.7* (8)	0.7 ± 0.8 (10)	4.0 ± 4.1 (11)

[a] Data presented are mean ± SD from number of experiments given in parentheses; * indicates $P < 0.05$.
[b] CE-I, extract before enzyme digestion, contains AFB_1 and its unconjugated metabolites. The total radioactivity of CE-I fraction is given in Table III.

Table V. Effect of BHA on the Preexisting Enzyme Activities during Hepatocyte Incubation[a]

Incubation condition	GSH-*S*-Transferase (nmol/min/mg)	UDP-glucuronyltransferase (nmol/min/mg)
Control	49 ± 6	1.2 ± 0.2
+BHA (0.25 m*M*)	48 ± 5	1.2 ± 0.2

[a] Cells were incubated with or without BHA for 60 min before microsomal and cytosolic fractions were isolated for the determination of transferase activities. Data presented are mean ± SD for four experiments.

eral mechanisms may be postulated to explain the observed protective effect of BHA. As an antioxidant, BHA may function as a free radical scavenger or may react with toxic intermediate as observed with other phenolic antioxidants (45). It is unlikely that a free radical is involved in the AFB_1 activation since TPA, a well known free radical scavenger (46), was ineffective in reducing AFB_1 content and binding in isolated hepatocytes (Table I) and in microsome-mediated binding of AFB_1 to calf thymus DNA (28). Another possible explanation could be that BHA increases the liver GSH level with GSH conjugating with the AFB_1-epoxide. It is also unlikely that GSH plays a major role in isolated hepatocytes since only a 32% inhibition of AFB_1 activation was observed in isolated hepatocytes (Table I). Furthermore, in BHA-treated cells, the formation of AFB_1 conjugates was not enhanced (Table IV). The lack of a major effect by GSH on AFB_1 activation in isolated hepatocytes reported from this laboratory (30) is in agreement with the results of Hayes *et al.* (47), who suggest that AFB_1 cytotoxicity studies in monolayer rat hepatocytes is independent of the GSH-dependent mechanism involved in cytoprotection. It is possible that BHA selectively inhibits cytochrome P-450 isozymes to reduce the formation of AFB_1-8,9-epoxide, consequently decreasing the availability of the reactive metabolites and their interaction with critical sites in DNA, RNA, or proteins. This possibility is supported by the following evidence: (i) results similar to those with BHA were obtained with known inhibitors of the cytochrome P-450 system, namely, metyrapone and SKF 525A (Tables I and II, Fig. 2); (ii) there were decreases in the formation of hydroxylated (AFM_1 and AFQ_1) and demethylated (AFP_1) polar metabolites and the accumulation of unreacted AFB_1 (Tables III and IV); (iii) there was a lack of effect by BHA on preexisting glutathione *S*-transferase and UDP-glucuronyltransferase (Table V) and on the level of conjugated forms of AFB_1 metabolites (Tables III and IV); and (iv) there was inhibition of cytochrome P-450 activities such as *O*-demethylation and AFB_1 activation (Table II and Figs. 1 and 3).

Although much information has been obtained about AFB_1 activation, formation of AFB_1-DNA, and enzymes involved in AFB_1 detoxication, considerably less is known about the process involved in the entry of AFB_1 into and transport through the cytoplasmic compartments, and the nuclear translocation of AFB_1. Induction of detoxifying enzymes by dietary BHA may be partially responsible for the observed reduction of cytotoxicity and tumor formation *in vivo* (3–9, 22–25), but the studies with isolated hepatocytes and cell-free systems suggest that inhibition of microsomal, cytochrome P-450-dependent monooxygenases may also contribute to the effect of the phenolic antioxidant. Thus, BHA acts at multistages in modulating carcinogenesis.

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1. Wattenberg LW. Chemoprevention of cancer. Cancer Res **45**:1–8, 1985.
2. Hoeman G. Chemoprevention of cancer: Phenolic antioxidants (BHT, BHA). Int J Biochem **20**:639–651, 1988.
3. Wattenberg LW, Coccia JB, Lam LKT. Inhibitory effects of phenolic compounds on benzo(*a*)pyrene-induced neoplasia. Cancer Res **40**:2820–2823, 1980.
4. McCormick, DL, Major N, Moon RC. Inhibition of 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinogenesis by concomitant or postcarcinogen antioxidant exposure. Cancer Res **44**:2858–2863, 1984.
5. Tatsuta M, Mikuni T, Taniguchi H. Protective effect of butylated hydroxytoluene against induction of gastric cancer by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in Wistar rats. Int J Cancer **32**:253–254, 1983.
6. William GM, Tanaka T, Maeura Y. Dose-related inhibition of aflatoxin B_1 induced hepatocarcinogenesis by the phenolic antioxidants butylated hydroxyanisole and butylated hydroxytoluene. Carcinogenesis **7**:1043–1050, 1986.
7. Benson AM, Batzinger RP, Ou SYL, Bueding E, Cha YN, Talalay P. Elevation of hepatic glutathione-*S*-transferase activities and protection against mutagenic metabolites of benzo(*a*)pyrene by dietary antioxidants. Cancer Res **38**:4486–4495, 1978.
8. Cha YN, Martz F, Bueding E. Enhancement of liver microsome epoxide hydratase activity in rodents by treatment with 2(3)-tert-butyl-4-hydroxyanisole. Cancer Res **38**:4496–4498, 1978.
9. Benson AM, Cha YN, Bueding E, Heine HS, Talalay P. Elevation of extrahepatic glutathione-*S*-transferase and epoxide hydratase activities by 2(3)-tert-butyl-4-hydroxyanisole. Cancer Res **39**:2971–2977, 1979.
10. Swenson DH, Miller EC, Miller JA. Aflatoxin B_1-2,3-oxide, evidence for its formation in rat liver *in vivo* and by human liver microsomes *in vitro*. Biochem Biophys Res Commun **60**:1036–1043, 1974.
11. Campbell TC, Hayes JR. The role of aflatoxin metabolism in its toxic lesion. Toxicol Appl Pharmacol **35**:199–222, 1976.
12. Gurtoo HL, Dahms RP. Effects of inducers and inhibitors on the metabolism of aflatoxin B_1 by rat and mouse. Biochem Pharmacol **28**:3441–3449, 1979.
13. Ishii K, Maeda K, Kamataki T, Kato R. Mutagenic activation of aflatoxin B_1 by several forms of purified cytochrome P-450. Mutat Res **174**:85–88, 1986.
14. Garner RC. Microsome-dependent binding of aflatoxin B_1 to DNA, RNA, polynucleotides and protein *in vitro*. Chem Biol Interact **6**:125–129, 1973.
15. Swenson DH, Lin JK, Miller EC, Miller JA. Aflatoxin B_1-2,3-oxide as a probable intermediate in the covalent binding of aflatoxin B_1 and B_2 to rat liver DNA and ribosomal RNA *in vivo*. Cancer Res **37**:172–181, 1977.
16. Lin JK, Miller JA, Miller EC. 2,3-Dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin B_1, a major acid hydrolysis product of aflatoxin B_1-DNA or -rRNA adducts formed in hepatic microsome-mediated reactions and in rat liver *in vivo*. Cancer Res **37**:4430–4438, 1977.
17. Essigmann JM, Croy RG, Bennett RA, Wogan GN. Metabolic activation of aflatoxin B_1: Patterns of DNA adduct formation, removal, and excretion in relation to carcinogenesis. Drug Metab Rev **13**:581–602, 1982.
18. Degan GH, Neumann H. The major metabolite of aflatoxin B_1 in the rat is a glutathione conjugate. Chem-Biol Interact **22**:239–255, 1978.
19. Neal GE, Colley PJ. The formation of 2,3-dihdyro-2,3-dihydroxy aflatoxin B_1 by the metabolism of aflatoxin B_1 *in vitro* by rat liver microsomes. FEBS Lett **101**:382–386, 1979.
20. Emerole GO, Neskovic N, Dixon RL. The detoxication of aflatoxin B_1 with glutathione in the rat. Xenobiotica **12**:737–743, 1979.
21. Hsieh DPH, Wong JJ. Metabolism and toxicity of aflatoxins. Adv Exp Biol Med **136**:847–883, 1981.
22. Lotlikar PD, Clearfield MS, Jhee EC. Effect of butylated hydrox-

yanisole on *in vivo* and *in vitro* hepatic aflatoxin B_1 DNA binding in rats. Cancer Lett **24**:241–250, 1984.
23. Kensler TW, Egner PA, Trush MA, Bueding E, Groopman JD. Modifications of aflatoxin B_1 binding to DNA *in vivo* in rats fed phenolic antioxidants, ethoxyquin, and a dithiothione. Carcinogenesis **6**:759–763, 1985.
24. Chang Y, Bjeldanes L. *R*-Goitrin- and BHA-induced modulation of aflatoxin B_1 binding to DNA and biliary excretion of thiol conjugates in rats. Carcinogenesis **8**:585–590, 1987.
25. Monroe DH, Eaton DL. Comparative effects of butylated hydroxyanisole on hepatic *in vivo* DNA binding and *in vitro* biotransformation of aflatoxin B_1 in the rat and mouse. Toxicol Appl Pharmacol **90**:401–409, 1987.
26. Pearson WR, Windel JJ, Morrow JF, Benson AM, Talalay P. Increased synthesis of glutathione-*S*-transferases in response to anticarcinogenic antioxidants. Cloning and measurement of messenger RNA. J Biol Chem **258**:2052–2062, 1983.
27. Sato K, Kitahara I, Yin Z, Waragai F, Nishimura K, Hatayama I, Ebina T, Yamazaki T, Tsuda H, Ito N. Induction by butylated hydroxyanisole of specific molecular forms of glutathione-*S*-transferase and UDP-glucuronyl-transferase and inhibition of development of γ-glutamyl-transpeptidase positive loci in rat liver. Carcinogenesis **5**:473–477, 1984.
28. Bhattacharya RK, Firozi PF, Aboobaker VS. Factors modulating the formation of DNA adduct by aflatoxin B_1 *in vitro*. Carcinogenesis **5**:1359–1362, 1984.
29. Firozi PF, Aboobaker VS, Bhattacharya RK. Modulation by certain factors of metabolic activation of aflatoxin B_1 as detected *in vitro* in a simple fluorimetric assay. Chem-Biol Interact **58**:173–184, 1986.
30. Ch'ih JJ, Lin T, Devlin TM. Activation and deactivation of aflatoxin B_1 in isolated rat hepatocytes. Biochem Biophys Res Commun **110**:668–674, 1983.
31. Berry MN, Friend DA. High yield preparation of isolated rat liver parenchymal cells. J Cell Biol **43**:506–520, 1969.
32. Lyman BA, Erki L, Biedrzycka DW, Devlin TM, Ch'ih JJ. Modification of protein synthetic components by aflatoxin B_1. Biochem Pharmacol **37**:1481–1486, 1988.
33. Ch'ih JJ, Devlin TM. The distribution and intracellular translocation of aflatoxin B_1 in isolated hepatocytes. Biochem Biophys Res Commun **122**:1–8, 1984.
34. Mathew CGP. The isolation of high molecular weight eukaryotic DNA. In: Walker JM, Ed. Methods in Molecular Biology. Clifton, NJ: Humana Press, Vol **2**: pp31–34, 1984.
35. Wei CI, Marshall MR, Hsieh DPH. Characterization of water-soluble glucuronide and sulphate conjugates of aflatoxin B_1. Urinary excretion in monkey, rat and mouse. Fd Chem Toxic **23**:809–819, 1985.
36. Yoshizawa H, Uchimaru R, Ueno Y. Metabolism of aflatoxin B_1 in the isolated nuclei of rat liver. J Biochem **89**:443–452, 1981.
37. Habig WH, Jakoby WB. Assays for differentiation of glutathione *S*-transferase. Methods Enzymol **77**:398–405, 1981.
38. Burchell B, Weatherill P. 4-Nitrophenol UDP-glucuronyltransferase (rat liver). Methods Enzymol **77**:169–177, 1981.
39. Kato R, Gillette JR. Effect of starvation on NADPH dependent enzymes in liver microsomes of male and female rats. J Pharmacol Exp Ther **150**:279–281, 1965.
40. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
41. Ch'ih JJ, Lin T, Devlin TM. Effect of inhibitors of microsomal enzymes on aflatoxin B_1-induced cytotoxicity and inhibition of RNA synthesis in isolated hepatocytes. Biochem Biophys Res Commun **115**: 15–21, 1983.
42. Cha YN, Heine HS. Comparative effects of dietary administration of 2(3)-tert-butyl-4-hydroxyanisole and 3,5-di-tert-butyl-4-hydroxytoluene on several hepatic enzyme activities in mice and rats. Cancer Res **42**:2609–2615, 1982.
43. Niranjan BG, Avadhani NG. Activation of aflatoxin B_1 by a monooxygenase system localized in rat liver mitochondria. J Biol Chem **255**:6575–6578, 1980.
44. Garner RC, Martin CN, Lindsay-Smith MR, Coles BF, Tolson MR. Comparison of aflatoxin B_1 and aflatoxin G_1 binding to cellular macromolecules *in vitro* and *in vivo* and after peracid oxidation: characterization of the major nucleic acid adducts. Chem Biol Interact **26**:57–73, 1979.
45. Chipman JK, Davis JE, Paterson P. Mechanisms of butylated hydroxytoluene-mediated modulation of 2-acetylaminofluorene mutagenicity in rat and human hepatocyte/salmonella assays. Mutat Res **187**:105–112, 1987.
46. Slater TF. Free Radical Mechanism in Tissue Injury. London: Pion Limited, p56, 1972.
47. Hayes MA, Murray CA, Rushmore TM. Influences of glutathione status on different cytocidal responses of monolayer rat hepatocytes exposed to aflatoxin B_1 or acetaminophen. Toxicol Appl Pharmacol **85**:1–10, 1986.