Effect of Butylated Hydroxyanisole Pretreatment on Aflatoxin B1-DNA Binding and Aflatoxin B1-Glutathione Conjugation in Isolated Hepatocytes from Rats

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ABSTRACT

The effect of 2(3)-tert-butyl-4-hydroxyanisole (BHA) pretreatment of rats on both aflatoxin B1, (AFB1)-DNA binding and AFB1-glutathione has been examined with isolated hepatocytes and in intact rats. Young male F344 rats were fed AIN-76A diet with or without 0.75% BHA for 2 weeks. Even though there were no significant differences in either cytochrome P-450 or reduced glutathione contents, there were marked differences in AFB1, metabolism in isolated hepatocytes from these two groups. Thus, at the 33 nM AFB1, level, AFB1-DNA binding was 3-fold higher in control compared to BHA-treated hepatocytes whereas AFB1-glutathione conjugation was 5-fold higher in treated compared to controls. Even at higher AFB1 concentrations (2 and 10 μM), DNA binding was 4-5-fold higher in controls whereas thiol conjugation was 5-9-fold higher in treated compared to control hepatocytes. Addition of 0.5–1 mm diethylnmaleate did not have any significant effect in control hepatocytes whereas its presence produced about 70–100% increase in DNA binding with corresponding decreases in thiol conjugation in treated hepatocytes. Addition of 1 mm styrene oxide caused 75% and 4-8-fold increase in AFB1-DNA binding in control and treated hepatocytes, respectively, with corresponding decreases in thiol conjugation. In intact rats, BHA treatment reduced hepatic AFB1-DNA binding to 15% of controls with concomitant increase in biliary excretion of AFB1-reduced glutathione conjugate. It appears that the induced cytosolic GSH S-transferases after BHA treatment of rats play a significant role in inhibiting hepatic AFB1-DNA binding and AFB1 hepatocarcinogenesis presumably by inactivation of the reactive AFB1-epoxide.

INTRODUCTION

Epidemiological data suggest that contamination of aflatoxin B1 in human food may be partly responsible for the induction of human liver cancer in regions of Africa and Asia (14). Antioxidants such as BHA, BHT, and ethoxyquin, which are widely used as preservatives in human and animal diets, inhibit carcinogenesis by various chemicals including AFB1, (5–8). AFB1, hepatocarcinogenesis is initiated by metabolic activation to generate the reactive AFB1-epoxide which then binds covalently to cellular DNA (9–11). Several laboratories including ours have shown that the microsomal cytochrome P-450 generated reactive AFB1-epoxide could be inactivated by cytosolic GSH S-transferases, presumably before the epoxide could enter the nucleus to interact with cellular DNA (12–18). Treatment of rodents with dietary antioxidants induces several phase II metabolizing enzymes including GSH S-transferases (19, 20). Our previous studies have demonstrated that hepatic cytosols from BHA-treated rats do inhibit microsomal-mediated AFB1 binding to either exogenous or endogenous DNA while they enhance AFB1-SG conjugation (21, 22).

In the present communication, we have extended these subcellular studies to freshly isolated intact hepatocytes from both control and BHA-treated rats. We have examined both AFB1-DNA binding and AFB1-SG conjugation during the metabolism of AFB1, and the effects of diethylnmaleate and styrene oxide on these reactions. The overall data show less AFB1-DNA binding and more AFB1-SG conjugation in hepatocytes from BHA-treated than in those from control rats, thereby further indicating that induction of GSH S-transferases by BHA is largely responsible for inhibiting hepatic AFB1-DNA binding and AFB1 hepatocarcinogenesis presumably by conjugation of the reactive AFB1-epoxide with GSH.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin, bovine pancreatic RNase, collagenase (type IV), β-glucuronidase (type H1), sulfatase (type H1), yeast-soluble RNA, calf thymus DNA, BHA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid, GSH, diethylnmaleate, DMSO, 5,5'-dithiobis(2-nitrobenzoic acid), and sodium dodecyl sulfate were purchased from Sigma Chemical Co. BHA consisting predominantly of 3-isomer (96%) with approximately 4% of 2-isomer was used without purification. Styrene oxide was obtained from Aldrich Chemical Co. HPLC grade CHCl3, methanol, and ethyl acetate were purchased from Fisher Scientific Co. Inhalation anesthetics, methoxyflurane, 2,2-dichloro-1,1-difluoroethyl methyl ether and halothane were purchased from Pitman-Moore, Inc., Washington Crossing, NJ, and Halocarbon Laboratories, Inc., Hackensack, NJ, respectively. Nonradioactive AFB1 and [3H]-AFB1 (specific activity, >30 Ci/mmol) were obtained from Moravek Biochemicals, Inc., Brea, CA. These compounds were used without further purification because they are stable in biological systems. Scintillation fluid (Liquisint) was obtained from National Diagnostics Company, Manville, NJ. Ca3Pb3Bondapak column (0.39 x 30 cm) and C18-Sep-Pak cartridges were obtained from Waters Associates, Inc., Emulgen 913 was kindly provided by Kao Atlas Chemicals, Tokyo, Japan. All other chemicals were of reagent grade.

Animals. Young male F344 rats weighing 100–125 g obtained from Charles River Breeding Laboratories, Wilmington, MA, were fed AIN-76A diet (23, 24) for 1 week before the animals were divided into two groups. One group of the AIN-76A diet fed for 2 more weeks and the second fed the AIN-76A diet containing 0.75% BHA for 2 weeks. All animals were given food and tap water ad libitum. Both AIN-76A diets were supplied in pellet form by Bioserve, Inc., Frenchtown, NJ, and stored at 4°C.

Isolation of Hepatocytes. After the rats were anesthetized by inhalation of methoxyflurane, hepatocytes were isolated by the collagenase method similar to that described previously (25). The preperfusion medium contained modified Hanks’ balanced salt solution without Ca2+ but containing 0.5 mm ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid-25 mM HEPES buffer (pH 7.4) and 0.5% serum albumin. This medium, saturated with 95% O2-5% CO2, was pumped at a rate of 15 ml/min (100 ml/animal). After preperfusion, the perfusion medium was pumped at the same rate as above. It contained modified...
Hanks' balanced salt solution containing 2 mM CaCl2-25 mM HEPES buffer (pH 7.4), 0.5% albumin, and 0.05% collagenase (250 ml/animal). The yield of hepatocytes was 3–4 x 10^8 cells/animal. Viability of cells was judged by the trypan blue exclusion method was found to be greater than 90%. These cells were used for various studies within 30 min after isolation.

Incubation of Hepatocytes. Duplicate portions of hepatocytes (2 x 10^7 cells/5 ml) were incubated in 25-ml polycarbonate Erlenmeyer flasks in the presence of modified Hanks' balanced salt solution containing 2 mM CaCl2-25 mM HEPES buffer (pH 7.4), 0.5% serum albumin, various levels of AFB1, (0.033, 2, and 10 µM) containing 1 µCi of [3H]AFB1 per ml, and various chemicals as indicated in 95% O2-5% CO2 at 37°C for 1 h. Sterylene oxide, AFB1, and DEM were dissolved in DMSO. The final concentration of DMSO was 2.0% (v/v). After incubation, flasks were immediately chilled in ice, cells were homogenized, and nuclei were sedimented at 600 x g for 10 min. Nuclear supernatants were used for the extraction, separation, and quantitation of AFB1-SG conjugate.

DNA Isolation and AFB1-DNA Binding. Isolation of DNA from the sedimented nuclei was as described previously (26). Isolated DNA was dissolved in 1 ml of 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA and portions were taken for colorimetric determinations of DNA, RNA, and protein by the published methods (27-29) and for radioactivity measurements. Using 5 ml of LiClis, samples were counted in a LKB 1219 Rackbeta liquid scintillation spectrometer with an efficiency of 45% for 3H counting. Protein and RNA contaminations in isolated DNA were <1%. Results of [3H]AFB1 binding to DNA are corrected for DNA recovery, which was 50–60%, and are expressed in terms of either pmol or nmol of AFB1 bound per mg DNA per h. Variations in duplicate samples were <5%.

Extraction, Separation, and Quantitation of AFB1-SG Conjugate. Nuclear supernatants were extracted twice with 2 volumes of CHCl3:ethanol (1:1, v/v). The nonextractable polar metabolites containing AFB1-SG conjugate were further purified by passing through a Q-Sep-Pak cartridge. After removal of unadsorbed material and radioactivity by washing the cartridge twice with 5 ml of H2O, AFB1-SG was eluted with 2 ml methanol. Portions of methanol concentrate were loaded on a HPLC C8 column (0.39 x 30 cm) and was eluted isocratically with 30% methanol in water containing 0.05% acetic acid at room temperature at a flow rate of 1 ml/min. During elution, absorbance at 365 nm was recorded and 1-ml fractions were collected for radioactivity measurements. Under these conditions, [3H]AFB1-SG was eluted with a retention time of 19 min, and radioactivity in three fractions between 18 and 20 min was corresponded to control AFB1-SG conjugate. Radioactivity in 1-ml fractions was determined using 5 ml of LiClis. Counting efficiency was about 45%. Results are expressed either as pmol or nmol of AFB1-SG conjugate formed/10^8 cells/h.

AFB1-SG conjugate standard was prepared by incubation of AFB1 in the presence of liver microsomes, NADPH, 5 mM GSH, and liver cytosol and purified by HPLC as described previously (30).

Examination of various experimental conditions revealed that with an incubation time of 2 h and cell density of 4 x 10^6/ml, conversions of 2 µM AFB1 to AFB1-DNA and AFB1-SG were linear for about 60–90 min. Therefore in all these studies, a density of 4 x 10^6 cells/ml and a 1-h incubation period were used.

Biliary Excretion of AFB1-SG Conjugate and Hepatic AFB1-DNA Binding in Vivo. For these studies, the bile duct was cannulated as described previously (31). Rats were anesthetized with a continuous flow of halothane and 95% O2-5% CO2 regulated by a Drager Vapor halothane vaporizer. A PE-50 polyethylene tube was inserted into the common bile duct up to the bifurcation and was firmly tied in place with silk ligatures. Once satisfied that the cannula was properly placed and bile flowed freely, the cannula was pulled through a skin tunnel in the presence of 0.2 M sodium acetate buffer, pH 5.5, and then homogenized in the same medium; and crude nuclear pellets were obtained by centrifugation of the homogenate at 600 x g. Isolation of DNA from the nuclear pellet and quantitation of AFB1-DNA binding were as described above for the hepatocyte system.

Extraction, separation, and quantitation of AFB1-SG in the bile were conducted as described above for the isolated hepatocyte system.

Miscellaneous Assays. The intracellular GSH in hepatocytes was determined colorimetrically with Ellman's reagent (32). Measurements were performed on 2 x 10^7 cells which were collected by gentle centrifugation at 100 x g for 5 min and treated with 5% trichloroacetic acid and, after 15 min in an ice bath, samples were centrifuged at 1200 x g for 10 min. Portions of supernatants were used for GSH analysis in the presence of 1 mM HEPES buffer, pH 8.3, using a calibration curve. For the analysis of cytochrome P-450, hepatocytes were solubilized in the presence of 0.08% Emulgen 913 containing 20% glycerol and 0.2 M potassium phosphate buffer, pH 7.4. Cytochrome P-450 content was then determined from the CO difference spectra of dithionite-reduced samples using an extinction coefficient of 91 mm1 cm^-1 between 450 and 490 nm (33).

RESULTS

Since AFB1-DNA binding and AFB1-SG conjugation are dependent on cytochrome P-450 and GSH levels, respectively (9, 17), it was necessary to determine cytochrome P-450 and GSH contents in isolated hepatocytes. Although cytochrome P-450 content was lower and GSH content was higher in hepatocytes from BHA-treated rats compared to their respective controls, the differences were statistically not significant (Table 1). Levels of GSH in both control and BHA-treated hepatocytes were lowered by 50–70% after 5 min. Incubation with 0.5–1.0 mM TEM (data not shown).

AFB1 metabolism in isolated hepatocytes displayed marked differences after BHA pretreatment (Table 2). At 33 nM AFB1, AFB1-DNA binding was one-third that in control, whereas AFB1-SG conjugation was 5-fold higher in treated than in controls. The presence of 1 mM TEM had no significant effect on AFB1-DNA or AFB1-SG formation in control hepatocytes whereas it nearly doubled AFB1-DNA binding and produced an 80% inhibition of thiol conjugation in BHA-treated hepatocytes. Sterylene oxide (1 mM) increased AFB1-DNA 4-fold and lowered AFB1-SG 87% in BHA-treated hepatocytes but increased AFB1-DNA and decreased AFB1-SG about 50% in control hepatocytes. At higher concentrations of AFB1 (2 and 10 µM), DNA binding was one-fourth to one-sixth of the control in the treated hepatocytes whereas thiol conjugation was 5-9-fold higher in the treated than in the control hepatocytes. At 0.5 mM TEM had no significant effect in control hepatocytes but increased DNA binding 70–100% and inhibited thiol conjugation 65% in treated hepatocytes. Sterylene oxide increased DNA binding 80–100% and lowered GSH conjugation 50–70% in control hepatocytes but increased DNA binding 5–9-fold and lowered GSH conjugation 80–90% in treated cells.

When hepatic AFB1-DNA binding and biliary excretion of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cytochrome P-450 and GSH contents in isolated hepatocytes from control and BHA-treated rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocytes</td>
<td>Cytochrome P-450 (nmol/10^8 cells)</td>
</tr>
<tr>
<td>Control</td>
<td>20.4 ± 1.1*</td>
</tr>
<tr>
<td>BHA-treated</td>
<td>17.9 ± 0.4*</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± SEM of three separate analyses on individual hepatocyte preparations.

a Data statistically not significant at P > 0.05 when compared with controls.
Metabolites were calculated for 10⁶ cells corresponding to 1 mg DNA/h. The results of this study on isolated hepatocytes and the liver carcinogenesis in the rat (7), it was obvious to infer that induced binding (12-18). Since pretreatment with BHA and various antioxidants induces hepatic GSH S-transferases in various species (19, 20) and also inhibits AFB₁ hepatic carcinogenesis in the rat (7), it was obvious to infer that induced GSH S-transferases may act by inhibiting AFB₁-DNA binding. The results of this study on isolated hepatocytes and the liver in intact rats support a role of BHA in prevention of carcinogenesis by modulating AFB₁-DNA binding. The reciprocal relationship between AFB₁-DNA binding and GSH-SG formation resulting from BHA treatment in rats clearly points to competition between the two processes for the available cytochrome P-450-activated AFB₁. By inducing high levels of GSH S-transferases, BHA directs AFB₁ to detoxication rather than DNA binding and carcinogenesis.

AFB₁-SG conjugate were examined in control and BHA-treated rats, marked differences were again observed (Table 3). Hepatic AFB₁-DNA binding was inhibited by about 85% whereas biliary excretion of AFB₁-SG conjugate was increased 3-fold in treated rats.

**DISCUSSION**

Previous studies have indicated that carcinogenic potency of AFB₁ in various species cannot be explained solely on its activation by cytochrome P-450 and that cytosolic GSH S-transferases play an important role in modulating AFB₁-DNA binding (12-18). Since pretreatment with BHA and various other antioxidants induces hepatic cytosolic GSH S-transferases in various species (19, 20) and also inhibits AFB₁, hepatocarcinogenesis in the rat (7), it was obvious to infer that induced GSH S-transferases may act by inhibiting AFB₁-DNA binding. The results of this study on isolated hepatocytes and the liver in intact rats support a role of BHA in prevention of carcinogenesis by modulating AFB₁-DNA binding. The reciprocal relationship between AFB₁-DNA binding and GSH-SG formation resulting from BHA treatment in rats clearly points to competition between the two processes for the available cytochrome P-450-activated AFB₁. By inducing high levels of GSH S-transferases, BHA directs AFB₁ to detoxication rather than DNA binding and carcinogenesis.

It has been demonstrated previously that hepatic AFB₁-DNA binding in the rat in vivo studies was essentially a linear function of AFB₁ dose when examined over a 10-20-fold concentrations (Ref. 6 and references therein). The present data on AFB₁-DNA binding indicate that this linearity is valid even in an isolated hepatocyte system over an even wider range of AFB₁ concentrations. In addition, BHA pretreatment exhibits an inhibitory effect on AFB₁-DNA binding over this entire range of AFB₁ concentrations by the formation of thiol conjugate via induced GSH S-transferases.

Further evidence for this concept derives from effects of DEM and styrene oxide. Intracellular GSH levels can be reduced in isolated hepatocytes and in vivo by pretreatment with DEM (34, 35), and hepatotoxicity of AFB₁ can be increased by DEM pretreatment of rats (36). In the present study, DEM treatment lowered intracellular hepatic GSH levels by 50-70% in hepatocytes from both groups. Although DEM treatment had no significant effect in control hepatocytes it increased AFB₁-DNA binding by 50-100% and lowered thiol conjugation 65-80% in BHA-treated hepatocytes. Like ethoxyquin-treated rats (37), even though there is a differential induction of constitutive GSH S-transferases by BHA treatment, it would appear from these results that GSH S-transferases induced by BHA differs from the enzymes in untreated control hepatocytes with different Kₘ. Such a possibility is being explored in our laboratory by purifying hepatic GSH S-transferases from both control and BHA-treated rats and examining their kinetic properties. Our DEM data with control hepatocytes are in agreement with those of Appleton et al. (35) who also reported no effect of DEM-pretreatment on hepatic AFB₁-DNA binding in intact Fischer rats.

Noninvolvement of epoxide hydrase as a modulator of AFB₁-DNA binding in subcellular experiments (16, 38, 39) enabled various epoxides to be used as valuable tools in examining their role in GSH S-transferase-mediated modulation of AFB₁-DNA binding (16, 17). Our previous subcellular studies in vitro, where GSH levels were high (5 mM), suggest that the increase of AFB₁-DNA binding and inhibition of AFB₁-SG conjugation in the presence of 1 mM styrene oxide were primarily due to inhibition of GSH S-transferases and not due to lowering of GSH levels (16, 21, 22). Similarly, in the present hepatocyte studies (Table 2), increased DNA binding and inhibition of thiol conjugation in the presence of styrene oxide suggest that the effect of styrene oxide is predominantly due to inhibition of GSH S-transferases. However, in view of lower GSH levels (2.0 mM) in hepatocytes (Table 1), some effect of styrene oxide by lowering hepatic GSH levels cannot be ruled out. A greater effect of styrene oxide in BHA-treated hepatocytes compared

### Table 3

**Effect of BHA pretreatment on hepatic AFB₁-DNA binding in vivo and biliary excretion of AFB₁-SG conjugate**

Male F344 rats were fed AIN-76A diet with or without 0.75% BHA for 2 weeks and bile ducts were cannulated as described previously (31) 18-24 h before sacrifice. Bile was collected for 2 h. Isolation and quantitation of hepatic nuclear DNA binding and extraction, separation, and quantitation of biliary excretion of AFB₁-SG were as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Animals</th>
<th>AFB₁-DNA binding (pmol/mg DNA) (%)</th>
<th>Biliary excretion of AFB₁-SG (pmol) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>440 ± 90 (100)</td>
<td>3.5 ± 0.5 (1.23 ± 0.18)</td>
</tr>
<tr>
<td>BHA-treated</td>
<td>469 ± 19 (16)</td>
<td>10.4 ± 1.5 (3.68 ± 0.33)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AFB₁ concentrations (nmol)</th>
<th>Additions</th>
<th>AFB₁-DNA (pmol)</th>
<th>AFB₁-SG (pmol)</th>
<th>AFB₁-SG/AFB₁-DNA</th>
<th>AFB₁-DNA (pmol)</th>
<th>AFB₁-SG (pmol)</th>
<th>AFB₁-SG/AFB₁-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>33 nm</td>
<td>DEM, 1 mm</td>
<td>9.1 ± 0.3</td>
<td>8.6 ± 0.5</td>
<td>0.95</td>
<td>3.2 ± 0.3</td>
<td>44.5 ± 0.7</td>
<td>13.9</td>
</tr>
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<td></td>
<td>SO, 1 mm</td>
<td>10.5 ± 0.6</td>
<td>6.9 ± 0.8</td>
<td>0.66</td>
<td>5.9 ± 0.5</td>
<td>9.0 ± 0.8</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>16.2 ± 0.8</td>
<td>4.3 ± 0.3</td>
<td>0.27</td>
<td>14.8 ± 1.2</td>
<td>5.9 ± 1.5</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>DEM, 0.5 mm</td>
<td>434 ± 18</td>
<td>265 ± 36</td>
<td>0.61</td>
<td>72 ± 15</td>
<td>1,415 ± 55</td>
<td>19.7</td>
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<td></td>
<td>SO, 1 mm</td>
<td>480 ± 25</td>
<td>210 ± 30</td>
<td>0.44</td>
<td>144 ± 10</td>
<td>490 ± 85</td>
<td>3.4</td>
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<tr>
<td></td>
<td>10 μM</td>
<td>860 ± 95</td>
<td>125 ± 30</td>
<td>0.15</td>
<td>660 ± 110</td>
<td>101 ± 23</td>
<td>0.15</td>
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<tr>
<td></td>
<td>DEM, 0.5 mm</td>
<td>973 ± 127</td>
<td>518 ± 102</td>
<td>0.53</td>
<td>232 ± 48</td>
<td>4,678 ± 657</td>
<td>20.2</td>
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<td></td>
<td>SO, 1 mm</td>
<td>1,068 ± 98</td>
<td>404 ± 63</td>
<td>0.38</td>
<td>390 ± 30</td>
<td>1,596 ± 372</td>
<td>4.09</td>
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<tr>
<td></td>
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<td>1,770 ± 227</td>
<td>148 ± 28</td>
<td>0.07</td>
<td>1,184 ± 252</td>
<td>370 ± 78</td>
<td>0.31</td>
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</table>

* Results are expressed as mean ± SEM on three separate analyses on individual livers.
* Data statistically not significant at P > 0.05 when compared with respective controls.
* Data statistically significant at P < 0.05 when compared with respective controls.

REFERENCES


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