Oltipraz-mediated Changes in Aflatoxin B1 Biotransformation in Rat Liver: Implications for Human Chemointervention

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ABSTRACT

Oltipraz (OPZ) is currently being considered for human use to protect against aflatoxin B1 (AFB)-induced hepatocarcinogenesis based on its proven protective effect in rats. The effectiveness of this treatment presumably that orthologous cytochrome P450 and glutathione S-transferase (GST) isozymes metabolize AFB in humans as they do in rats. In this study, alterations in the expression of multiple forms of cytochrome P450 and GST were evaluated after treatment with OPZ, as well as other known P450 inducers, including 3-methylcholanthrene, pregnenolone-16α-carbonitrile, and ciprofibrate. Evidence is presented that the male-specific rat CYP 3A2, an orthologue of human CYP 3A4, may be primarily responsible for AFB activation in rat liver at both high and low AFB substrate concentrations. The CYP 1A2 enzyme does not appear to play a role in AFB activation in rat liver at any substrate concentration, whereas the major human P450 enzyme capable of activating AFB at a low substrate concentration has been identified as CYP 1A2. Surprisingly, we found that the CYP 1A2 steady-state mRNA level and the CYP 1A2-associated methoxyresorufin-O-demethylase activity were induced approximately 3- to 2-fold, respectively, by OPZ in rat liver. However, because CYP 1A2 does not appear to participate in AFB activation, induction of CYP 1A2 may be insignificant for AFB-induced hepatocarcinogenesis in rat models. In the rat, a heterodimeric α-class GST enzyme containing the Yc2 subunit is the only polypeptide characterized to date in this species with high catalytic activity for the conjugation of activated AFB with glutathione. The GST Yc2 steady-state mRNA level was induced 5-fold by OPZ treatment. This induction was mirrored by significant increases in both the corresponding protein level and AFB-8,9-epoxide-conjugating enzyme activity, which may contribute significantly to protection against AFB-induced carcinogenesis in the rat. Investigations from this and other laboratories have not revealed any evidence for a Yc2-like GST isoform with high AFB-8,9-epoxide-conjugating activity in human liver. We have also been unable to demonstrate that the two major human α-class GST isozymes, A1-1 and A2-2, purified from bacteria expressing the corresponding cDNAs, exhibit any significant AFB-8,9-epoxide-conjugating activity. Our results suggest that humans may not be protected to the same extent as rats against AFB-induced hepatocarcinogenesis by treatment with OPZ and that further investigations are needed to establish the usefulness of OPZ for protection against human exposure to AFB.

INTRODUCTION

AFB4, a mycotoxin produced by the fungus Aspergillus flavus, is a very potent toxin and hepatocarcinogen (1). OPZ, a synthetic dithiolethione derivative previously used as an antischistosomal agent (2), has been shown to protect laboratory animals from the hepatocarcinogenic effects of AFB (3–6). OPZ is also an effective radioprotector and chemoprotector against carbon tetrachloride and acetaminophen toxicity (2). In laboratory animals, OPZ induces Phase II drug-metabolizing enzymes, most notably GSTs (7–9). Steady-state rat GST Ya-type mRNA levels are induced 3-fold by OPZ (0.075% in the diet), and regulation appears to be at the transcriptional level (7). In the rat, dietary OPZ treatment produced a 3.2-fold induction of the GST Ya2 subunit but only a 1.2-fold induction of the GST Ya1 subunit (8). Other induced GST subunits include Yc2 (subunit 10, 2.4-fold) and Yf (subunit 7, 4.6-fold; Ref. 8). Very recently, Primiano et al. (13) reported the induction of GST subunits by OPZ in F344 rats. These authors found a preferential induction of the GST Ya2, Yc2, Yb1, and Yf subunits of 5–11-fold over control. Clapper et al. (14) reported a preferential induction of GST μ class protein and mRNA transcripts by OPZ in female ICR mice, together with an intermediate induction of GST class π and a moderated induction of the GST catalytic θ protein and mRNA transcript levels. OPZ preferentially induced mRNAs encoding the α class human GST subunits A1 and A2 in primary human hepatocyte cultures (9), with varying effects on the GST M1 level and no effect on the GST P1 level.

In several areas of the world, humans are exposed to AFB in their diet, and it is generally considered to be both an animal and a human carcinogen (15). However, across species, a large variability in susceptibility toward AFB-induced hepatocarcinogenesis has been observed (15). Mice were found to be resistant, and hamsters were moderately resistant, whereas rats, and potentially humans, readily develop liver cancer on exposure to AFB (1). AFB is metabolically activated in the liver by the microsomal cytochrome P450 system to the reactive AFBO, which has been demonstrated to bind to proteins and nucleic acids, including DNA. AFB-DNA adducts, in the form of AFBO\(^{-N\prime}\)-guanine, can be measured in urine as markers for AFB exposure (16). Recently, our laboratory has shown that two P450 enzymes with different kinetic characteristics can metabolize AFB in human liver (CYP 1A2, with a relatively low apparent \(K_m\), and CYP 3A4, with a relatively high apparent \(K_m\). For AFB; Ref. 17). Therefore, at the low AFB concentrations encountered in human diets, metabolic activation of AFB is catalyzed predominantly by CYP 1A2 in most individuals. Only at relatively high doses of AFB, or in humans with very low levels of CYP 1A2 and high levels of CYP 3A4, are significant amounts of AFB converted to the active AFBO by CYP 3A4. Although human CYP 3A4 is capable of activating AFB to the epoxide, the predominant metabolite of CYP 3A4 is the detoxification product AFQ (17–19). There is evidence suggesting that P450 3A2 (orthologous to human CYP 3A4) and 2C11 may be involved in the metabolic activation of AFB in the rat (20, 21).

The major detoxification mechanism for AFBO in rodents is by conjugation with GSH, catalyzed by GSTs (1). Mice are apparently protected from AFB-induced hepatocarcinogenesis because of the relatively high constitutive expression of a hepatic GST isozyme

Received 8/21/95; accepted 3/15/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This research was supported by NIH Grant ES-05780. T. M. B. was a recipient of Swiss National Science Foundation Fellowship 823 A-033323. Presented at the 85th Annual Meeting of the American Association for Cancer Research, Inc.

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The abbreviations used are: AFB, aflatoxin B1; AFQ, aflatoxin Q1; OPZ, oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione]; GST, glutathione S-transferase; AFBO, AFB-8,9-oxide; GSH, reduced glutathione; BHA, butylated hydroxyanisole; CF, ciprofibrate; EQ, ethoxyquin; PCN, pregnenolone-16α-carbonitrile; MROD, methoxyresorufin-O-demethylase; 3MC, m-methylcholanthrene; HPCL, high-performance liquid chromatography; EROD, ethoxyresorufin-O-deethylase.

5 The GST subunits are identified by the nomenclature for rat isozymes proposed by Bass et al. (10). For further reference on nomenclature, see Mantle et al. (11). The human GST subunits are identified by the nomenclature for human GST isozymes proposed by Mannervik et al. (12).
OLITIPRAZ-MEDIATED CHANGES IN AFB BIOTRANSFORMATION

(GST Yc) with high catalytic activity for AFB, and can be induced by treatment with phenolic antioxidants such as BHA or EQ. Pretreatment of rats with BHA, EQ, or OPZ protects rats from developing liver cancer when exposed to AFB, and it is presumed that this effect is a result of increased detoxification due to elevated expression of the GST Yc subunit (3, 8, 24–27).

Based on the effectiveness of OPZ in protecting rats from the hepatocarcinogenic effects of AFB, studies were initiated to use OPZ as a chemoprotective agent in Phase II clinical trials in AFB-exposed humans in the Qidong region in China (28). Inasmuch as the major protective effect of OPZ in rats can be attributed to the induction of a GST Yc2-containing isozyme, more research is needed to determine whether such an activity may be present and/or inducible in humans. In addition, more information regarding the possible effects of OPZ on the expression of cytochrome P450 enzymes involved in AFB oxidation is needed in both rats and humans. We present here evidence that OPZ treatment of rats induces CYP 1A2 mRNA and enzyme activity. However, this induction is of no consequence in this rat model, because we also show that, in contrast to the human enzyme, rat CYP 1A2 does not appear to be capable of activating AFB. Our results indicate that CYP 3A2 appears to be the major rat P450 enzyme capable of activating AFB to AFBO at both high and low AFB substrate concentrations. In addition, our results support the hypothesis that the major protective effect of OPZ in rats is afforded by the induction of the GST Yc2 isozyme with high catalytic activity toward AFB.

MATERIALS AND METHODS

Chemicals. PCN was obtained from Upjohn Co. (Kalamazoo, MI). 3MC and 3,4-dichloro-1-nitrobenzene were from Aldrich Co. (Milwaukee, WI). OPZ [5-(2-pyrazinyl)-4-methyl-1-2-dithiole-3-thione] was generously provided by Dr. Thomas W. Kensler (Department of Health Sciences, The Johns Hopkins University, Baltimore, MD). Tris base, GSH, NADPH, glucose-6-phosphate dehydrogenase, 2,3-tert-butyl-4-hydroxyanisole (mixed isomers), EQ, diethylpyrocarbonate, formamide, and β-mercaptoethanol were from Sigma Chemical Co. (St. Louis, MO). Emulphor EL-719 was from GAF Co. (New York, NY). Ethanol was obtained from Midwest Grain Products Co. (Pekin, IL). Agarose (ultrapure electrophoresis grade), guanidinium isothiocyanate (enzyme grade), acid phenol (redistilled nucleic acid grade), and some other molecular biology reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). T4 polynucleotide kinase was purchased from Stratagene (La Jolla, CA). [γ-32P]ATP was purchased from DuPont NEN Research Products (Boston, MA). The GST Yc2 antibody was prepared as described previously (29). HPLC-grade solvents were obtained from J. T. Baker (Phillipsburg, NJ). Other reagents were of analytical reagent grade and were obtained from various commercial sources.

GST Clones and Proteins. GST proteins were expressed in Escherichia coli using the vectors pET17b (Novagen, Inc., Madison, WI) for the expression of mouse Yc-Yc and rat Yc2-Yc2 and pKK5 (a variant of the vector pKK223–3; Pharmacia, Piscataway, NJ) for the expression of all other recombinant GSTs used in this study, and the expressed proteins were purified to homogeneity using GST affinity matrices. Mouse GST Ya and Yc2 cDNAs were cloned by Buetler and Eaton (30). The GST Yc2 gene was cloned by Buetler and Eaton (30). The GST Yc2 gene was cloned by Buetler and Eaton (30). The GST Yc2 gene was cloned by Buetler and Eaton (30). The GST Yc2 gene was cloned by Buetler and Eaton (30).

Animals and Treatments. Adult male Sprague-Dawley rats (approximate 200 g) were housed in plastic cages on wire mesh-covered corn cob bedding. Rats were maintained for 1 week on a Wayne Rodent Blox chow diet and then switched to a purified AIN-76A (Teklad Research Diets, Madison, WI) diet for 1 week. Food and water were available ad libitum. In experiment 1, four animals received i.p. injections of single doses of PCN (75 mg/kg); three animals received injections of 3MC (20 mg/kg) in a vehicle of 73% isotonic saline, 25% corn oil, and 2% Emulphor EL-719; and six control animals received vehicle injections only. All animals were euthanized 24 h after treatment. In experiment 2, OPZ [0.075% (w/w)] and CF [0.025% (w/w)] were administered daily for 3 days in the AIN-76A diet to four animals in each of the treatment groups and six animals in the control group. Animals were euthanized by CO2 narcosis and exsanguination. Livers were removed, rinsed in ice-cold PBS, partially minced, immediately frozen in liquid nitrogen, and stored at -80°C. Some of these animals and treatments were also used as positive controls for two additional studies reported elsewhere (33, 34).

Tissue Preparations. Part of the frozen liver tissue was used to prepare cytosolic and microsomal fractions, as described previously (35), for Western blot and enzyme activity analysis.

Oligonucleotide and cDNA Probes. The amino acid sequences of the known rat GST isozymes were aligned using the Clustal software program (36, 37). Unique amino acid stretches were identified, and the corresponding cDNA sequences served as templates for the design of antisense oligonucleotides of 20 nucleotides in length. Each GST-specific probe contained at least three mismatches to other known nontarget rat GST sequences. Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Milligen/Biosearch, Burlington, MA) and purified by HPLC. The sequences of the gene-specific oligonucleotides were: sequence encoding GST Yc1, ATGCTCTCGGTGTA-CATGTG; encoding GST Yc2, ATACCTCTGTAACTAGTCTG; encoding GST Yc1, GGGAATTTGTCACAAAGCCT; encoding GST Yc2, GTCCAA-TAGCTCGTGGTCCA; encoding GST Yb1, CCTCAAGGGGACTC-GCA; encoding Yb2, CAGACACTGCAAACTGTAG; encoding Yf, GTC- GGATGGCGGCGGCTT; encoding CYP 1A2, GCAAAGGACCT-GGGCC; encoding CYP 2C11, TACCCGACGACGAGTCTC; encoding CYP 3A2, CAGACAGGCTTGTAGACAGAC; and encoding CYP 4A1, CCAATCTCCGCGACTGTCG. Gene-specific oligonucleotides recognizing the cytochrome P450 enzymes were obtained from Dr. Curt Omiecinski (University of Washington). An oligonucleotide directed against 18S rRNA was used as an internal control for RNA loading and was also provided by Dr. Omiecinski.

RNA Isolation and Northern Blots. Ten or 20 mg total RNA were separated on a 1.25% agarose/formaldehyde gel, blotted onto Nytran membranes, and hybridized with 32P-end-labeled oligonucleotides. Northern blots were hybridized in buffer [5× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate), 1× Denhardt’s solution, 0.1% SDS, 1× EDTA, and 10 mg/μl] containing 0.1 mg/ml denatured salmon sperm DNA for 18 h at 50–56°C (depending on the oligonucleotide) in a rotating hybridization oven (Robbins Scientific, Sunnyvale, CA). Blots were rinsed once in wash buffer [5× SSC and 0.1% SDS] at room temperature, followed by two washes in wash buffer for 15 min at hybridization temperature. Autoradiograms were scanned and quantitatively analyzed using a Biotek 3000 densitometric scanner and Millipore’s Bioimage whole-band analysis software (Milligen/Biosearch, Bedford, MA).

Western Blot Procedures. Cytosolic proteins were separated on a 1.0 mm SDS polyacrylamide gel (16% acrylamide:0.09% N,N-bis-acrylamide) and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Nonspecific binding was blocked with 0.5% I-block (Tropix, Bedford, MA) in PBS. Primary antibodies were incubated overnight in 0.1% I-block in PBS containing 0.1% Tween 20. Blots were then incubated with a secondary antirabbit alkaline phosphatase-conjugated antibody (Bio-Rad, Richmond, CA) for 4 h. Blots were developed using a color substrate mix (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium development kit; Bio-Rad) for alkaline phosphatase.

Enzyme Assays. The microsomal oxidation of AFB was measured as described previously using microsomes of rats treated with the different enzyme inducers (35). Incubations were allowed to proceed for 10 min at 37°C in the presence of excess mouse liver cytosol to trap all AFBO formed (19). To measure cytosolic GST AFBO-conjugating activity, microsomes from BHA-treated mice that produce high levels of AFBO were incubated for 10 min at

6 T. M. Buetler, H. S. Ramsdell, and D. L. Eaton, unpublished data.
37°C with cytosolic fractions from rats treated with enzyme inducers. The amount of cytosol added was adjusted to ensure the linearity of the assay conditions. Formation of AFB metabolites was detected by HPLC as described previously (35, 38). The enzymatic activity toward the general GST substrate 1-chloro-2,4-dinitrobenzene was determined spectrophotometrically as described by Habig and Jakoby (39).

RESULTS

AFBO-conjugating Activities in the Mouse, Rat, and Human. AFBO-conjugating activities of purified GSTs were measured, using mouse microsomes as the AFBO-generating system (Table 1). Of all the bacterially expressed, homodimeric α class GSTs measured (mouse Ya-Ya and Yc-Yc; rat Yα2-Yα2, Yc1-Yc1, and Yc2-Yc2; and human A1-1 and A2-2), only the mouse Yc-Yc and the rat Yc2-Yc2 isozymes exhibited significant activities toward AFBO (results for mouse Ya-Ya, rat Yα2-Yα2, and human A2-2 are not shown). The rat Yc2-Yc2 and mouse Yc-Yc enzymes displayed approximately 120- and 640-fold higher AFBO activity, respectively, compared with the closely related rat Yc1-Yc1 protein. None of the μ class (mouse Yb2-Yb2 and rat Yb1-Yb1 and Yb2-Yb2), or π class (mouse Yf-Yf) GSTs examined were found to exhibit any significant AFBO-conjugating activity (results not shown). The subunits encoded by the rat Yc2 and mouse Yc cDNAs share 91% sequence homology with each other but only 75–76% with the three human α class GST subunits, A1–A3. Interestingly, although the rat Yc1 protein is highly homologous to both the rat Yc2 (90% homology) and mouse Yc (85% homology) subunits, the Yc1-Yc1 protein has less than 1% AFBO-conjugating activity displayed by these isozymes.

Induction of GST and CYP Isozymes by OPZ. Induction of GST mRNAs by OPZ was quantitated by densitometric scanning of Northern blots hybridized with gene-specific oligonucleotide probes. RNA loading was normalized by quantitation of the hybridization signal with an 18S rRNA oligonucleotide (29). Fig. 1 shows the relative fold induction calculated as the ratio of the averaged normalized hybridization signal of four treated animals versus that of control animals. Consistent with reports by Davidson et al. (7), Meyer et al. (8), and Primiano et al. (13), we found that the GST Yα2 mRNA was induced 3-fold, whereas the GST Yα1 mRNA was not significantly induced (Fig. 1). The GST Yc1 mRNA was also induced approximately 3-fold by OPZ. On average, OPZ treatment resulted in a 5-fold induction of GST Yc2 mRNA, although there was some interanimal variability (Figs. 1 and 2). For example, one of four OPZ-treated rats showed little induction of Yc2, and one of six controls showed significant constitutive expression of Yc2. GST Yb1 mRNA was also induced significantly (4-fold), but expression of GST Yb2 and Yf mRNAs was not affected by OPZ. The induction of the GST Yc2 protein was confirmed by Western blotting analysis (Fig. 2), using an antibody raised against mouse GST Yc (25) that cross-reacts with rat GST Yc2, Yc1, and Ya-type subunits (Yα1 and Yα2). Each lane represents mRNA or protein isolated from an individual animal. OPZ (Lanes 1–4 on the Northern blot correspond to OPZ Lanes 1–4 on the Western blot).

Table 1 AFBO-conjugating activity of bacterially expressed GSTs

<table>
<thead>
<tr>
<th>Proteins expressed in E. coli</th>
<th>Purified by GSH-agarose affinity chromatography and analyzed by the standard HPLC method for AFBO (32) and spectrophotometry for 1-chloro-2,4-dinitrobenzene (CDNB) (36). Values are the mean of n number of experiments ± SE.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFBO generated by mouse liver microsomes (nmol/min/mg)</td>
<td>CDNB (μmol/min/mg)</td>
</tr>
<tr>
<td>GST enzyme</td>
<td></td>
</tr>
<tr>
<td>Mouse YcYc</td>
<td>192.5 ± 11.5</td>
</tr>
<tr>
<td>n = 6</td>
<td>n = 6</td>
</tr>
<tr>
<td>Rat Yc2Yc2</td>
<td>35.4 ± 5.1</td>
</tr>
<tr>
<td>n = 6</td>
<td>n = 6</td>
</tr>
<tr>
<td>Rat Yc1Yc1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>n = 3</td>
<td>n = 3</td>
</tr>
<tr>
<td>Human A1-1</td>
<td>0.06 ± 0.00</td>
</tr>
<tr>
<td>n = 3</td>
<td>n = 3</td>
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</table>

* Practical detection limit, −0.1 nmol/min/mg.

Fig. 1. Induction of GST mRNA by OPZ. Bars, relative induction of mRNA in OPZ-treated versus control animals. Northern blots were hybridized with gene-specific oligonucleotides for individual GST subunit genes. Densitometric scans of hybridization signals were normalized to signals obtained from hybridization with an 18S rRNA oligonucleotide. Each bar represents the average relative induction of four animals. * OPZ-induced subunit mRNA was significantly different from that of control animals at P < 0.05; †, GST Yf is not constitutively expressed in male rat liver.

Fig. 2. Induction of GST Yc2 mRNA and protein by OPZ. Top, Northern blot probed with a GST Yc2-specific oligonucleotide; bottom, Western blot probed with an antibody raised against the mouse Yc protein cross-reacting with the rat Yc1, Yc2, and Yα. The location of the GST Yc2 protein is indicated. Lane 1, mouse GST Yc; Lane M, M, 28,000 protein standard. Each lane represents RNA or protein isolated from an individual animal. OPZ Lanes 1–4 on the Northern blot correspond to OPZ Lanes 1–4 on the Western blot.
TABLE 2

| AFB concentrations (Table 2). These results suggest that rat CYP 3A enzymes are involved in the formation of both AFBO and AFQ, similar to CYP 3A4 in human liver microsomes (19). 3MC, a CYP 1A family-selective inducer, also caused elevated formation of both AFBO and AFQ (2–3-fold). However, this effect is most likely mediated not by CYP 1A2 (mRNA induced 25-fold) but by CYP 3A2, the mRNA of which was induced 2-fold by 3MC (Fig. 5). The CYP 1A-associated EROD activity was induced 44-fold by 3MC (33). Interestingly, OPZ treatment caused a significant 3-fold increase in the CYP 1A2 mRNA level, which was accompanied by a significant 55% increase in CYP 1A2-selective MROD activity (Fig. 3). It should be noted that these changes were not mirrored by an increase in the formation of either AFBO or AFQ (Table 2). In fact, OPZ treatment resulted in a slight, albeit insignificant, decrease in the formation of AFBO at the high AFB substrate concentration and a significant decrease at the low AFB substrate concentration. Thus, in contrast to the observations in human liver microsomes, rat liver CYP 1A2 does not appear to participate in the metabolic activation of AFBO at either AFB substrate concentration.

Induction of Cytosolic AFBO-conjugating Activity by OPZ. Treatment with OPZ produced a significant 1.8-fold increase in the rate of formation of the GSH-AFB conjugate, which is most likely mediated by GST Yc2 (Table 3). The 1.2-fold increase in the formation of the GSH-AFB conjugate in animals treated with CF was not statistically significant.

Relative Ratio of Activation over Inactivation of AFB in OPZ-treated Rats. Activation was calculated as the rate of AFB metabolized to AFBO divided by the rate of total oxidative AFB metabolites formed (AFQ + AFBO; formation of AFM or AFP was not observed). The rate of inactivation was calculated as the fraction of total AFBO conjugated with GSH (40). The ratio of activation over inactivation is presented in Fig. 6. These calculations support the supposition that rats treated with OPZ are protected from AFB carcinogenicity because they have a lower activation:inactivation ratio than untreated rats.

Fig. 6. Relative ratio of activation over inactivation of AFB in OPZ-treated rats. Bars, se. The mean is the average activity of all animals analyzed. MROD activity in OPZ-treated rats is significantly different statistically from control animals at P < 0.05.
The formation of AFBO and AFQ in microsomes of treated animals was analyzed as described (16) using high (128 μM) and low (16 μM) AFB concentrations. The AFBO formed was trapped by a mixture of mouse and rat liver cytosol containing excess GSTs and the glutathione conjugate, representative of the amount of AFBO formed, and AFQ was observed for the induction of AFQ and induction of CYP 1A2 and 3A2 mRNAs encoding specific P450 enzymes (CYP 1A2, 2C11, 3A2, 3A4) at both low and high AFB substrate concentrations in rats of mRNAs encoding specific P450 enzymes (CYP 1A2, 2C11, 3A2, 3A4) at both low and high AFB substrate concentrations. Several lines of evidence suggest that rat CYP 1A2 does not contribute significantly to the activation of AFB to AFBO. First, the 2-3-fold increase in the rate of AFBO formation in rats treated with the CYP 1A inducer 3MC to the activation of AFB to AFBO. First, the 2-3-fold increase in the rate of AFBO formation correlates well with the CYP 3A2 mRNA induction supports the argument that CYP 3A2 is the principal P450 enzyme in the rat liver responsible for the oxidative metabolism of AFB to both AFBO and AFQ at both high and low AFB substrate concentrations.

**DISCUSSION**

The carcinogenic mycotoxin AFB is activated to the DNA-binding electrophile AFBO by the microsomal cytochrome P450 system. In the human liver, CYP 1A2 and 3A4 are the two major P450 enzymes capable of catalyzing this reaction (17). Human CYP 1A2 appears to be more active at low AFB substrate concentrations, which are more representative of dietary exposure. In contrast, CYP 3A4 contributes more to the formation of AFBO at high AFB substrate concentrations. In addition, CYP 3A4 may play an important role in the activation of AFB in individuals expressing low levels of CYP 1A2 but high levels of CYP 3A4 (17). CYP 3A4 is also responsible for the formation of the detoxification product AFQ in human liver. In contrast to humans, our results indicate that in rat liver, CYP 3A2 (the rat orthologue of human CYP 3A4) represents the major enzyme forming AFBO and AFQ at both high and low AFB substrate concentrations. Several lines of evidence suggest that rat CYP 1A2 does not contribute significantly to the activation of AFB to AFBO. First, the 2-3-fold increase in the rate of AFBO formation in rats treated with the CYP 1A inducer 3MC does not correlate with the large increase (40-fold) in CYP 1A-associated EROD activity (Table 2; Refs. 33 and 41); however, the observed 2-3-fold induction in AFBO formation correlates well with the 2-fold induction of CYP 3A2 mRNA in rats treated with 3MC (Fig. 5). Second, if CYP 1A2 were capable of activating AFB at low substrate concentrations, a poor correlation between CYP 3A2 mRNA induction and AFBO formation would be expected at low AFB substrate concentrations; however, the correlation coefficient was almost the same at both low (R2 = 0.96) and high AFB substrate concentrations (R2 = 0.97; Fig. 7).

In this study, we did not analyze the induction of CYP 3A1, the other major rat P450 isoform of the 3A family. Circumstantial evidence suggests that CYP 3A1 may contribute little to the metabolism
of AFB, because CYP 3A1 is not constitutively expressed in either the male or female rat liver, whereas CYP 3A2 is constitutively expressed in the male rat liver (42). Using microsomes prepared from an untreated male rat liver, we observed a significant formation of AFBO (Table 2). In contrast, when microsomes from a female rat liver were used, we were unable to detect any formation of AFB oxidation products in our standard AFB assay. However, we did not investigate the effect of PCN treatment on AFB metabolism in female rats (in female rats, PCN induces only CYP 3A1 but not 3A2; see Ref. 42). Interestingly, female rats are less susceptible than male rats to the hepatocarcinogenic effects of AFB (1). Therefore, it is tempting to speculate that the relative resistance of female rats compared with male rats may be explained, at least partly, by: (a) the lack of expression of the P450 3A2 enzyme that is largely responsible for AFB activation; and (b) the expression of significant levels of the detoxifying GST Yc2 isozyme. However, additional experiments in female rats treated with PCN are required to evaluate the role of CYP 3A1 in AFB metabolism.

Our finding that both CYP 1A2 mRNA and the associated MROD activity were induced 3- and 1.5-fold, respectively, by OPZ in the rat liver (Figs. 3 and 4) raises the important question of whether OPZ has a similar effect on human hepatic CYP 1A2 expression. In rats, this induction is unlikely to have an impact on hepatocarcinogenesis, because we show here that the rat hepatic CYP 1A2 enzyme does not appear to participate significantly in the activation of AFB (Fig. 7). However, because the CYP 1A2 enzyme appears to be primarily responsible for AFB activation in the human liver at low (dietary) AFB concentrations, a 2-3-fold induction of CYP 1A2 may significantly increase the rate of AFBO formation in OPZ-treated humans. Indeed, the data by Morel et al. (9), using primary human hepatocytes in culture, show in at least one human liver sample (HL 5) a slight but visible induction of CYP 1A2 mRNA by OPZ treatment. Although these are ex vivo data, they lend some support to our current hypothesis.

The protective effect of OPZ preventing AFB-induced hepatocarcinogenesis in rats is believed to be due, at least in part, to increased detoxification of activated AFB by GST isoenzymes containing the Yc2 subunit. Our observations that OPZ treatment of rats generally results in approximately a 5-fold induction of the GST Yc2 mRNA, an increase in the associated protein, and a 1.8-fold increase in the AFBO-conjugating activity in liver cytosol (Fig. 2 and Table 3) are consistent with this hypothesis. However, it should be noted that there was some interanimal variability in this response, and it is possible that some animals may remain refractory to OPZ-mediated GST Yc2 induction. Nevertheless, on average, calculation of the theoretical ratio of activation/inactivation shows a lower ratio in OPZ-treated rats than in untreated rats (Fig. 6). This effect is due to an increased detoxification of activated AFB that is probably mediated by GST.
class GST isozymes (Al-1 and A2-2) have little or no activity to
identified. Alternatively, OPZ may provide a different mechanism of
any significant protection from AFB-induced carcinogenesis in hu
human Al-1 and A2-2 in our standard AFB assay, it appears that
human hepatocytes caused an increase in the mRNA and protein
i.e., mouse Yc-Yc and rat Yc2-Yc2.
from the only two isozymes with high AFBO-conjugating activity,
ious human GST isozymes cloned to date are significantly different
human cytosol, our results show that the two major human liver a
isozymes (Al-1 and A2-2) were below the detection limit of our
microsomally generated AFBO, respectively, compared with rat cy-
tosol (Table 1; Ref. 43). Furthermore, the AFBO-conjugating activi-
ties of the two bacterially expressed, recombinant human α class GST
isozymes (Al-1 and A2-2) were below the detection limit of our
assay, even when 100–150 times the amount of the rat GST Yc2 or
mouse GST Yc, respectively, was used (Table 1). Microsomes from
different organisms vary in the ratio of formation of the two stereoi-
somers exo- and endo-AFBO (20). Because only the exo-AFBO
steroisomer appears to be capable of DNA binding and causing
mutations (44), and because the mouse liver microsomes used in this
study almost exclusively form the exo-AFBO stereoisomer (43), there
seems to be no constitutively expressed GST enzyme capable of
efficiently conjugating the more harmful exo-AFBO stereoisomer in
the normal human liver. The fact that OPZ can induce human GST µ
class transcripts in primary hepatocyte cultures (9) is of less signifi-
cance in this context, because the human µ class GST (GST M1a-
M1a) appears to be capable of conjugating only the endo-epoxide of
AFBO, which has a low DNA-binding capacity (20). In addition to
human cytosol, our results show that the two major human liver α
class GST isozymes (Al-1 and A2-2) have little or no activity to
conjugate exo-AFBO. Moreover, the amino acid sequences of the
three human GST isozymes cloned to date are significantly different
from the only two isozymes with high AFBO-conjugating activity,
*e.g.* mouse Yc-Yc and rat Yc2-Yc2.

Recently, Morel et al. (9) showed that OPZ treatment of primary
human hepatocytes caused an increase in the mRNA and protein
levels of human GST A1-1 and A2-2. Because we detected little, if
any, AFBO-conjugating activity associated with bacterially expressed
human A1-1 and A2-2 in our standard AFBO assay, it appears that
induction of these two human GST isozymes by OPZ may not provide
any significant protection from AFBO-induced carcinogenesis in hu-
mans. However, we cannot exclude the possibility that OPZ may
induce the expression of nonconstitutively expressed human liver
GST isozyme(s) possessing AFBO-conjugating activity not yet iden-
tified. Alternatively, OPZ may provide a different mechanism of
protection in human liver not involving GST enzymes. Such a mech-
anism may be provided by aldehyde reductase (45).

Based on the anticarcinogenic effect of OPZ in rats, Phase II
clinical trials have been initiated in the Qidong region in China, where
individuals are exposed to high dietary doses of AFB and, conse-
quently, run a high risk of developing liver cancer. Our data show that
AFB biotransformation is different in humans and rats. The rat is at
least partially protected against AFBO-induced hepatocarcinogenesis
by OPZ treatment, because this compound induces GST Yc2-Yc2
possessing high AFBO-conjugating activity. It is proposed that OPZ
treatment lowers the activation/inactivation ratio, which results in
decreased AFB-DNA binding and a reduced incidence of formation of
mutations, neoplasia, and cancer of the liver in rats. The rat orthologue
of the P450 enzyme capable of activating AFB at low substrate
concentrations in humans (CYP 1A2) does not appear to play a role in
AFB activation in the rat liver. Our results raise the possibility that
OPZ-mediated chemointervention in humans may induce the level of
CYP 1A2, the enzyme primarily responsible for AFBO activation at low
AFB substrate concentrations. In addition, OPZ may not be as effec-
tive in protecting humans against the hepatocarcinogenic effects of
AFB, because, to date, there is no experimental evidence indicating
the existence of a human GST gene encoding a protein with high
exo-AFBO-conjugating activity. Therefore, it is possible that OPZ
 treatment may result in an increased activation of AFBO, without
affording protection by increasing GST-mediated detoxification.
Therefore, caution should be exercised in implementing long-term
human intervention studies using OPZ until the efficacy and safety of
this treatment can be confirmed with short-term studies in humans
and/or nonhuman primates.

**ACKNOWLEDGMENTS**

The excellent technical assistance of Dennis Slone is gratefully acknowl-
edged. We also thank Dr. Curt Omieciński for providing the CYP oligon-
ucleotides and for helpful discussions. Dr. Alan J. Townsend for providing
the mouse GST-µ1 and -µ2 expression clones, Drs. Cecil Pickett and Thomas
Rushmore for providing the cDNA clone for rat GST Ya2 and Yc1, Dr. David
Tu for providing the purified human GST A1-1 and A2-2 proteins, and
Dr. Thomas Kessler for providing OPZ.

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