ABSTRACT

Dithiolethiones are thought to act as potent chemoprotective agents against aflatoxin B1 (AFB1)-induced hepatocarcinogenesis in the rat by inducing glutathione S-transferases (GSTs). To determine whether these antioxidants can be similarly effective in human beings, we have investigated metabolism of AFB1 in primary human hepatocytes with and without pretreatment by oltipraz (OPZ), a synthetic derivative of the natural 1,2-dithiole-3-thione. Aflatoxin M₁ (AFM₁), glutathione conjugates of AFB₁ oxides (AFBSGs), and unchanged AFB₁ were quantitated in cultures derived from eight human liver donors. Parenchymal cells obtained from the three GST M₁-positive livers metabolized AFB₁ to AFM₁ and to carcinogenic exo-epoxide. From the three GST M₁-positive livers metabolized AFB₁ to AFM₁ and to AFBSGs, and unchanged AFB₁ were quantitated in cultures derived from eight human liver donors. Parenchymal cells obtained from the three GST M₁-positive livers metabolized AFB₁ to AFM₁ and to AFBSGs derived from the ischemic exo- and endo-8,9-oxides, whereas no AFBSGs were formed in the GST M₁-null cells. Pretreatment of the cells with 3-methylcholanthrene or rifampicin, inducers of CYP1A2 and CYP3A4, respectively, caused a significant increase in AFB₁ metabolism. Although OPZ induced GST A2, and to a lesser extent GST A1 and GST M₁, it decreased formation of AFM₁ and AFBSG, which involves CYP1A2 and CYP3A4. Inhibition by OPZ of AFB₁ metabolism by reducing CYP1A2 and CYP3A4 was also demonstrated by decreased activity of their monooxygenase activities toward ethoxyresorufin and nifedipine, respectively. The significant inhibition by OPZ of human recombinant yeast CYP1A2 and CYP3A4 was also shown. These results demonstrate that AFBSG can be formed by GST M₁-positive human hepatocytes only, and suggest that chemoprotection with OPZ is due to an inhibition of activation of AFB₁, in addition to a GST-dependent inactivation of the carcinogenic exo-epoxide.

INTRODUCTION

AFB₁, a product of the mold Aspergillus flavus, which infests grain and other food stuff stored under warm, moist conditions, is metabolized by certain CYPs to a powerful genotoxin, exo AFBO (1–3). AFB₁ is therefore a potential carcinogen; however, its carcinogenicity varies from one species to another (4). In the mouse, its hepatocarcinogenicity is negligible (5); this has been attributed to the particular effective detoxication of exo AFBO by the GST Yc-Yc (6, 7). On the other hand, it is a powerful hepatocarcinogen in the rat apparently due to the absence, in any quantity, of a GST that utilizes toward exo AFBO (11) and no apparent homologue for rat GST 10 (10, 11), the rat homologue of mouse GST Yc.

AFB₁-associated carcinogenesis (8, 9), apparently by inducing expression of GST 10 (10, 11), the rat homologue of mouse GST Yc.

It has been assumed that AFB₁ is also a human hepatocarcinogen because hepatocellular carcinoma is common in many communities that consume AFB₁-contaminated diets (12). Recently, this association was clearly established by an epidemiological study showing that AFB₁ is not only a hepatocarcinogen per se in humans, but is also a powerful cocarcinogen in combination with hepatitis B virus, which is often endemic in communities exposed to AFB₁ (13).

The question arises whether inducers, so effective in the rat, might be of value for the chemoprevention of the AFB₁-associated hepatocellular carcinogenesis in humans. The potential for GST-dependent detoxication in the human liver is not as great as in the rat liver because human liver has about 1 order of magnitude less GST activity toward exo AFBO (11) and no apparent homologue for rat GST 10–10 and mouse GST Yc-Yc. The most active GST toward exo AFBO is human GST M₁–1 (14), the benefit of which is limited by a genetic polymorphism involving a null allele, so abundant that about 50% of the human population has a GST M₁-null phenotype (15).

Human hepatocytes in primary culture have proved to be useful in past studies of GST induction (16). In the present work, the use of hepatocytes in culture has been extended to studies of the effects of inducers on the metabolism of AFB₁. Particular importance is attached to OPZ, a pharmaceutical derived from D3T, which has been reported to be a natural product occurring in cruciferous vegetables (17). In human hepatocytes, OPZ proves to be not only an inducer of GSTs but also an inhibitor of exo AFBO biosynthesis by CYP1A2 and CYP3A4. The mechanism of this effect has been investigated, and the significance of these findings for chemoprevention of AFB₁-dependent hepatocellular carcinoma in humans is discussed.

MATERIALS AND METHODS

Chemicals. Culture media and FCS were obtained from GIBCO (Paisley, Scotland). Collagenase, MC, RIF, bovine albumin, bovine insulin, AFB₁, and AFBO, metabolites were products from Sigma Chemical Co. (St. Louis, MO). D3T was synthesized by Dr T. W. Kensler, and OPZ was kindly supplied by Dr C. G. Caillard (Rhône-Poulenc Rorer, Antony, France).

Cell Isolation and Culture. Human liver samples were obtained in France from eight patients undergoing liver resection for primary or secondary hepatomas (Table 1). Hepatocytes were isolated by a two-step collagenase perfusion procedure, and the experimental procedures used were approved by the National Ethics Committee (18). Cell viability was 70–85%, as estimated by the trypan blue exclusion test. Cell yields varied with the size of the liver fragment, and the design of each experiment (time points and treatments) depended on the number of viable cells obtained.

Liver parenchymal cells were seeded at a density of 10⁶ viable cells/35-cm² dish in 2 ml of a nutrient medium consisting of 75% MEM and 25% Medium 199, supplemented with 10 μg bovine insulin/ml, 0.2% BSA, and 10% FCS. This medium, supplemented with 0.1 μM dexamethasone but lacking serum, was renewed daily.

Inducers were added 36–48 h after cell seeding and then 24, 48, and 72 h later, with medium renewal. MC, D3T, OPZ, and RIF were dissolved in DMSO before addition to the culture medium to give final concentrations of 5
CYP3A4 cDNAs were expressed in yeasts as described by Renaud et al. (23). The pyridine was measured in the supernatant by HPLC according to Guengench et al. (22). Oxidation of nifedipine to its metabolites was measured in cultures refreshed with MEM without phenol red and containing nifedipine at a concentration of 20 μM. The pyridine was measured in the supernatant by HPLC according to Guengench et al. (22).

GST Subunit Analysis. The cells were sonicated, and a total GST fraction was obtained from the supernatant by GSH agarose affinity chromatography. GST subunits were quantified from the absorbance at 214 nm. Cellular protein concentration was determined using the Bradford method (20). Total CYP concentrations were determined by UV spectroscopy according to the method of Prough et al. (26). Samples of yeast microsomes containing 50 pmol of CYP1A2 were incubated at 28°C with variable concentrations of NADPH (0.2—1.6 μM) in 0.1 M potassium phosphate buffer. The reaction was initiated by the addition of 125 μM NADPH. Fluorescence was measured directly in the cuvettes after stirring for 2 min. Calibration was made by addition of 53 pmol of resorufin to the reaction medium.

AFB1 Metabolism by Untreated Hepatocytes. The use of fluorescence analysis allowed determination of AFB1 metabolism without the need for additional reactions or chemical manipulations. AFB1 metabolism was quanitified by HPLC in untreated cells after 2—5 days of culture and 8 h of exposure to the mycotoxin.
Table 2  Induction of the GST subunits by OPZ in human hepatocytes in primary culture after 48 h of treatment

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>M1a</th>
<th>A1</th>
<th>A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-1</td>
<td>C</td>
<td>0.38</td>
<td>1.80</td>
<td>0.52</td>
</tr>
<tr>
<td>HL-5</td>
<td>OPZ</td>
<td>0.80(2.10)</td>
<td>3.90(2.17)</td>
<td>2.40(4.00)</td>
</tr>
<tr>
<td>HL-6</td>
<td>OPZ</td>
<td>1.38(1.64)</td>
<td>6.20(1.77)</td>
<td>4.20(1.90)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.23</td>
<td>1.70</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>OPZ</td>
<td>0.33(1.43)</td>
<td>6.50(3.82)</td>
<td>1.50(2.14)</td>
</tr>
</tbody>
</table>

The number of time points examined was determined by the size of the liver sample, smaller samples limiting the number. A 3-day culture period appeared to give a good representation of metabolism, and this was chosen for comparative studies.

Effect of GST M1 Polymorphism. Three metabolites were identified in the GST M1-positive liver cells (i.e., AFM1 and the GSH conjugates of endo and exo AFBO); these two isomers were produced in the same proportion (Table 1). In contrast, only AFM1 was formed in GST M1-negative hepatocyte cultures.

Effect of OPZ on AFB1 Metabolism and GST Expression. In all cases, OPZ-treated hepatocytes had lower AFB1 metabolism than their untreated counterparts after either 3, 4, or 5 days of culture. Less AFB1 was metabolized, and less AFBSG and AFM1 were produced after 48 h of treatment. In the three GST M1-positive samples, AFBSG was negligible in one case and reduced by 71 and 84% in the others. The production of AFM1 was negligible in 4 cases and decreased by 82–97% in the others (Fig. 1). A similar decrease in AFB1 metabolism was observed when hepatocytes were exposed to the parent D3T. To determine whether the decrease in AFB1 biotransformation observed in the culture medium was not due to the retention of metabolites or unmetabolized AFB1 in the hepatocytes, cell homogenates were also assayed. Only negligible amounts of AFB1 and its metabolites were detectable in either untreated or OPZ-exposed hepatocytes. Neither AFB1 nor its metabolites were adsorbed into the plastic culture dishes used.

We have already shown that OPZ increased human GST A1, A2, and to a lesser extent, GST M1 transcripts (16). This was reevaluated at the protein level for the three GST M1-positive hepatocyte populations used in the present study after a 48-h exposure to OPZ. As shown in Table 2, OPZ increased GST A1 subunit 2.0-, 1.8-, and 3.8-fold; GST A2 by 4.0-, 1.9-, and 2.1- fold; and GST M1 by 1.6-, 1.4-, and 2.0-fold in liver samples HL-1, HL-5, and HL-6, respectively.
had a greater affinity for and was consequently a more potent competitive inhibitor of CYP1A2 in comparison with CYP3A4.

Preincubation of OPZ with microsomes in presence of a NADPH-generating system for 5 min before adding the substrate in the same conditions as above enhanced inhibition. The percentage of inhibition was 71 ± 6%, with 1.6 µM ethoxyresorufin and 10 µM OPZ without preincubation, whereas it reached 85 ± 7% after a 5-min preincubation of the microsomes with OPZ (results not shown). In the presence of 50 pmol expressed CYP3A4 microsomes/ml, 100 µM testosterone, and 100 µM olitipraz, the percentage of inhibition varied from 6 ± 1% without preincubation to 47 ± 6% after a preincubation with OPZ. These results support the conclusion that OPZ is not only a competitive inhibitor of CYP1A2 and CYP3A4 substrates but also acts partly as an irreversible inhibitor.

Effect of MC and RIF on AFB₁ Metabolism in the Presence or Absence of OPZ. The decreased metabolism of AFB₁ in OPZ-treated culture suggested that OPZ had a direct effect on biotransformation by CYP1A2 and CYP3A4. To address this hypothesis, hepatocyte cultures were exposed to MC and RIF, specific inducers of CYP1A2 and CYP3A4, respectively. As shown in Fig. 2, MC treatment resulted in a marked increase in both metabolism of AFB₁ and formation of AFBSG and AFM₁. RIF also increased AFBSG levels but did not affect AFM₁ formation, and had only a moderate positive effect on overall AFB₁ metabolism. When coincubated with MC, OPZ partly suppressed formation of the two metabolites, whereas coincubation with RIF caused almost complete inhibition of increase of AFB₁ metabolism seen with RIF alone.

Effect of Exposure to OPZ on Cellular Activities of CYP1A2 and CYP3A4. OPZ given to cells in primary culture inhibited EROD and nifedipine oxidation, two activities catalyzed by CYP1A2 and CYP3A4, respectively (Fig. 3), whereas 4-fold inductions of both EROD by MC and nifedipine oxidation by RIF were observed after 2 days. Simultaneous incubation with OPZ resulted in a marked decrease in both CYP-related enzyme activities.

Effect of OPZ on the Enzyme Kinetics on Heterologously Expressed CYP1A2 and CYP3A4. CYP1A2 and CYP3A4 were expressed in yeasts and microsomal preparations from the recombinants used to study the inhibition kinetics of OPZ. Dixon plots were constructed that showed that during the 30-min incubation period, OPZ behaved as a competitive inhibitor of both enzymes (Fig. 4). Respective Kₛ, calculated from the reciprocal plots of velocity versus OPZ concentration, were 10 and 80 µM for CYP1A2 and CYP3A4, respectively. The 8-fold lower Kₛ for CYP1A2 indicates that OPZ
min⁻¹·mg⁻¹; GST A2–2: 0.02 nmol min⁻¹·mg⁻¹; and GST M1–1, 0.6 nmol min⁻¹·mg⁻¹, whereas for the endo AFBO, the values are nil for both GST A1–1 and GST A2–2, but 1.4 nmol min⁻¹·mg⁻¹ for GST M1–1 (14). GST M1–1 is more active than the others by at least 1 order of magnitude. Our results fit with this difference because appreciable AFBSG formation occurred only in hepatocytes expressing GST M1. Because human hepatocytes in primary culture formed both endo and exo AFBSG, and the exo-oxide has been shown to react DNA very efficiently and cause mutation (28), the lack of GST M1 activity in GST M1-null individuals may be of considerable consequence. Our observations also support and explain the study of Liu et al. (29), who reported that human liver cytosol obtained from GST M1-deficient individuals (GST M1 null) was less protective against AFM1-induced mutagenicity in the Ames Salmonella assay than liver cytosol from GST M1-positive individuals. The major role played by GST M1–1 in conjugation of AFBO with GSH in humans and by GST 10–10 in the rat is another example of a major discrepancy in behavior between laboratory animals and humans. No equivalent of the rat GST 10–10 is induced de novo in human liver.

Our study has focused on quantitation of AFBO conjugates and AFM1, but these metabolites represent only about 10% of the total AFB1 biotransformation. Most of the metabolites appeared to be excreted because after an 8-h incubation, no intracellular accumulation of AFB1 or AFM1 metabolites could be detected. Further studies are directed toward identification of other metabolites produced by human hepatocytes, including AFQ1. This metabolite, formed by 3α-hydroxylation of AFB1, by CYP3A4, is regarded as a detoxication product and is known to have low acute toxic and carcinogenic effects, if any (30). In recent years, considerable attention has been given to the development of chemointervention strategies involving induction of GSTs, with activity toward exo AFBO and a possible decrease in the extent of DNA damage in populations with high aflatoxin exposure. OPZ has been proposed as a good candidate because of its protective effect against AFM1-induced hepatocarcinogenesis in the rat (17). OPZ is known to be an inducer of GSTs and of some other enzymes, such as quinone reductase (31). In a previous study, we demonstrated that this compound also induces GSTs in human hepatocytes in primary culture at the mRNA level. We confirm these observations with other human cell populations at the protein level and the higher response of GST-α compared to GST-µ class. OPZ increased GST A1 by 4-fold and GST A2 and GST M1 by 2-fold. This induction effect was not associated with an increased formation of AFBSG; this was overridden by an inhibitory effect of OPZ on AFM1, metabolism reduction of CYP1A2 and CYP3A4 activities. It is not a general effect on CYPs because some other CYPs, such as CYP2C8, were not affected by the antioxidant (data not shown). In addition, this inhibitory effect is not species specific because similar CYP inhibition by OPZ occurred in rat hepatocytes. It should be emphasized that OPZ was used at a concentration comparable to that in plasma from humans treated with a single dose of 500 mg of OPZ (32), therefore validating the extrapolation of these in vitro data to the in vivo situation.

In GST M1-positive cells, AFBSG production, which depends on both CYP1A2 and CYP3A4, was reduced by as much as 84% after treatment with OPZ. AFM1 production, which depends solely on CYP1A2, was decreased by as much as 97%. The use of specific inducers for these two CYPs confirmed previous results, showing the implication of CYP1A2 and CYP3A4 in the biotransformation of AFB1 (33). Thus, by inducing CYP1A2, MC increased the formation of AFM1 and AFBSG in our cells, whereas by inducing CYP3A4, RIF enhanced formation of AFBSG.

The inhibitory effect of OPZ on both CYP1A2 and CYP3A4 was confirmed by two other approaches: (a) in hepatocytes, EROD and nifedipine oxidation, which are catalyzed by CYP1A2 and CYP3A4, respectively, were decreased in OPZ-treated cells; and (b) experiments with recombinant enzymes expressed in yeasts show that this inhibition is partly due to a competition between the AFB1 and OPZ for CYP1A2 and CYP3A4, and also partly to an irreversible inhibition by OPZ of these two CYPs. There may, however, be another element in inhibition observed in hepatocytes, particularly with respect to CYP3A4, because OPZ reduced its activity to very low levels whether the substrate was AFB1 or nifedipine, and much lower than would be expected for a Kᵢ as high as 80 μM. The possibility that the hepatocytes metabolize OPZ to a more potent inhibitor is under study. These effects have also been observed with D3T, a natural compound, which is present in extracts of cruciferous vegetables, indicating that various dithiolethiones can have the same inhibitory effect.

To our knowledge, this is the first demonstration of an inhibitory effect of OPZ on human CYP activities. Such an effect had been evoked by Putt et al. (34) when studying metabolism of AFM1 in OPZ-treated rats. It could be concluded that some shortcomings that might arise from low activities of GSTs or, even in GST M1-null individuals, the absence of any GST-dependent detoxification could be compensated for in terms of chemoprotection by inhibition of activation by Phase I enzymes. It must be borne in mind, however, that without Phase I enzymes, AFM1 cannot undergo appreciable detoxication by CYP, although reduction to aflatoxicol followed by gluturonidation is an alternative mechanism.

REFERENCES


Inhibition of CYP1A2 and CYP3A4 by Oltipraz Results in Reduction of Aflatoxin B₁ Metabolism in Human Hepatocytes in Primary Culture

Sophie Langouët, Brian Coles, Fabrice Morel, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/55/23/5574

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.