Effects of administration of the chemoprotective agent oltipraz on CYP1A and CYP2B in rat liver and rat hepatocytes in culture

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The success of oltipraz (OPZ) [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione] as a chemoprotective agent against aflatoxin B1 (AFB1)-induced hepatocarcinogenesis in the rat is thought to depend principally on its ability to enhance detoxication by inducing phase II enzymes, especially glutathione transferases. However, in primary cultures of human hepatocytes, we recently demonstrated that OPZ also has an important inhibitory effect on the major cytochromes P450 (CYPs) of human hepatic AFB1 metabolism. This has prompted a detailed study of the effect of OPZ on some CYPs involved in metabolism of AFB1 in the rat. Primary cultures of rat hepatocytes behaved similarly to human hepatocytes and responded to OPZ by inhibition of ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-depentylase (PROD) activities mainly associated, respectively, with CYP1A and CYP2B. A time-course shows that this inhibition is largely reversible, with EROD and PROD activities reaching a minimum at 12 h and tending towards control values within 24 h. As is to be expected, the incubation of isolated microsomes with OPZ also inhibits CYP1A and 2B. The effect of OPZ on CYP1A is not a phenomenon limited to cells in culture, but also occurs in vivo. Using the whole animal, we were able to demonstrate that OPZ also transiently inhibited CYP1A activity in a rat given caffeine, by measuring the amounts of methylxanthines found in the serum. However, microsomes isolated from rats, that had been treated with OPZ in vivo, show no such inhibition, presumably because, since OPZ is a reversible inhibitor, it dissociates and is lost during the course of conventional procedures of microsomal preparation. This explains some earlier failures in studies of isolated microsomes to observe the inhibition of CYPs by OPZ. In addition to inhibiting their enzymatic activity, OPZ is also an inducer of CYP1A and 2B as shown by the increased levels of their mRNAs and of caffeine metabolism in vivo after 24 h or more. It is concluded that the mechanism of chemoprotection by OPZ, of toxic chemical metabolism in the rat, is complex and involves competitive inhibition of activation succeeded by induction of the enzymes of both activation and detoxication.

Introduction

A number of natural and synthetic compounds, that protect against chemical carcinogenesis, have been proposed to do so by the induction of enzymes of detoxication such as the phase II enzymes: glutathione transferases (GSTs*), epoxide hydroxylase, NADP(H): quinone reductase and uridine 5'-diphosphate (UDP)-glucuronosyl transferases (1–4). One such agent is oltipraz (OPZ) [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione], a synthetic derivative of the plant product 1,2-dithiole-3-thione [2-(pyridyl)ethyl glutathione], which has proved effective in a number of animal models of experimental carcinogenesis (5), including aflatoxin B1 (AFB1) induced hepatocarcinogenesis in the rat (1,6,7). In this case protection against AFB1 toxicity has been associated with induction of alpha class GSTs, particularly GST 10-10, an enzyme normally exclusive to the perinatal liver (8–11). The expectation that OPZ might provide similar protection against AFB1-associated hepatocarcinogenesis in man (12) is undergoing clinical trial in the district of Qidong, near Shanghai. In this area hepatocellular carcinoma is associated with both a high dietary intake of AFB1 and a high incidence of hepatitis B, each of which is a human hepatocarcinogen and, in combination with the other, a powerful co-carcinogen (13).

In the human, drug metabolism can, to a limited degree, be followed by analysis of metabolites in the blood and urine. However, in order to test directly the effects of OPZ on all the enzymes that might be affected in AFB1 metabolism, isolated human hepatocytes in primary culture have been used as a surrogate. In these hepatocytes it was shown that, while OPZ elevates human hepatic GSTs, AFB1 conjugation, rather than being increased, is markedly decreased. This was the result of inhibition by OPZ of the cytochrome P450s, CYPA2 and CYP3A4, the principal enzymes of AFB1 activation in man (14). This challenges the currently accepted view that the mechanism of protection by OPZ against AFB1-induced hepatocarcinogenesis in the rat is primarily based on GST induction.

In the present study, we have investigated the effects of OPZ on cytochrome P450’s (CYPs) in rat liver both in vivo and in vitro and shown that, as in man, OPZ is an inhibitor of CYPs involved in AFB1 activation. OPZ is also shown to be an inducer of enzymes of activation in addition to enzymes of detoxication. We also demonstrate why earlier experimental
approaches using microsomes isolated from OPZ-treated rats have not detected OPZ inhibition of AFB1 activation (15).

Materials and methods

Chemicals

Culture media and fetal calf serum were from Gibco (Paisley, Scotland); collagenase, 3-methylcholanthrene (MC), phenobarbital (PB), bovine albumin, bovine insulin, caffeine, AFB1 and AFB2 metabolites from Sigma Chemical Co. (St Louis, MO). 1,2-Dithiole-3-thione (D3T) was synthesized by Dr T.J.Curphey (Dartmouth Medical School, Hanover, NH), and OPZ was kindly supplied by Dr C.G.Caillard (Rhone-Poulenc Rorer, Antony, France). All other compounds were readily available commercial products of the highest purity available.

Animal treatment

Male Sprague–Dawley rats (200 g) were treated i.p. with MC (20 mg/kg per day) injected in solution in corn oil (2 ml/kg) for 3 or 5 days (16), or with PB (80 mg/kg) injected in phosphate-buffered saline (PBS) for 16 h, or treated with OPZ, which was incorporated into the diet (A04, Centre d’Elevage Janvier, Genest, France) at a final concentration of 0.075% (w/w) and fed for 1–5 days (1). In the studies of in vivo caffeine metabolism OPZ was administered by gavage at a dose of 100 mg/kg per day. At least three animals were used for each experimental condition.

Cell isolation and culture

Isolated hepatocytes were obtained by perfusion of the liver of Sprague–Dawley rats (200 g) with a collagenase solution as previously described (17). Cell viability, estimated by the Trypan Blue exclusion test, was found to range between 90–95%. The cells were seeded at a density of 10⁷ hepatocytes/80 cm² flask in 10 ml of standard medium consisting of 75% minimum essential medium and 25% minimum essential medium and 25% medium 199, containing 0.2 mg/ml bovine serum albumin, 10 mg/ml bovine insulin and 10% fetal calf serum. Four hours after hepatocyte seeding, a serum-free medium containing 10⁻⁷ M dexamethasone and 10 mM nicotinamide was added to the cultures. The four inducers, PB, MC, OPZ and D3T, were added to culture media 24 h after cell seeding and thereafter with each daily renewal of the culture media. MC and OPZ, dissolved initially in dimethylsulfoxide (0.2%, w/v), were added to the culture medium so that the final concentrations of MC was 5 µM and OPZ and D3T 50 µM. PB, dissolved in PBS, was added to the culture medium to give a concentration of 3.2 mM.

CYP1A1 and CYP2B1/2 activities in rat hepatocytes and in rat microsomes

In hepatocytes ethoxyresorufin O-deethylase (EROD), associated with CYP1A1 and 2, and pentoxyresorufin O-depentylase (PROD), associated with CYP2B1/2 as well as with CYP2C11, CYP26 and CYP3A2 (18), were measured essentially as described by Burke and Mayer (19), reaction rates being determined under linear conditions with regard to incubation time and protein concentration (20).

In microsomes EROD and PROD were measured at 37°C according to Tauc et al. (21), a method derived from Prough et al. (22).

RNA isolation and Northern blot analysis

Total RNA was extracted from livers and cultured hepatocytes by the method of Chirgwin et al. (23), as modified by Raymondejean et al. (24). RNA (10 µg) was subjected to electrophoresis in a denaturing formaldehyde/agarose gel and transferred onto Hybond-N+ sheets. The sheets were pre-hybridized and hybridized with the following 32P-labeled probes. The different probes used are P-450 C6 complementary to CYP 1A1 mRNA (25), pR17 complementary to rat CYP2B2 mRNA (26) and a specific oligonucleotide for CYP2C11 mRNA (27). After hybridization, filters were washed (2x saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS)) to 0.1x SSC, 0.01% SDS), dried and autoradiographed at −80°C. Filters were then stripped of the previous probes and rehybridized with nick translated genomic 18S probes (28) used as controls. Relative amounts of mRNA were determined by densitometry (Densilab, Microvision Instruments, Evry, France).

Analysis of AFB1 metabolites

Rat hepatocytes were incubated with 5 µM AFB1 for 8 h after various times of exposure to the inducers. Both media and cells were then separated and stored at −80°C until use. AFB1 metabolites were analysed by high pressure liquid chromatography (HPLC) as described previously (14).

Caffeine metabolism in rats and in hepatocyte cultures

Rats were given 25 mg/kg caffeine, dissolved in water, by gavage. Blood was collected by heart puncture after 8 h and centrifuged at 4000 g for 10 min at 4°C. The supernatant corresponding to the serum was then stored at −80°C. Kidney and liver after cell seeding, hepatocytes were incubated with 10⁻⁷ M caffeine for 24 h in the absence of fetal calf serum. As previously reported (29), caffeine at this concentration does not give rise to toxicity as judged by either light microscopy or lactate dehydrogenase leakage. After incubation, culture media and cells were collected and stored at −80°C.

Sera from in vivo experiments or media from hepatocyte cultures were extracted with 2 vol. of chloroform–isoamyl alcohol (85:15, v/v) after saturating the aqueous phase with ammonium sulfate. The organic phase was separated, evaporated at 40°C under a stream of nitrogen and then the caffeine metabolites theobromine (TB), paraxanthine (PX) and theophylline (TP) were separated and quantitated by HPLC according to Berthou et al. (30).

Results

Effects of OPZ on CYP activities in microsomal preparations

With the intention of determining whether OPZ behaves as an inhibitor of CYP activities in rat liver, EROD and PROD activities were measured in microsomal preparations from animals treated for 3 or 5 days with this compound. No reduction of EROD and PROD activities was observed; on the contrary, both activities were augmented by a factor of 1.5 to 2 (Figure 1). However, this increase was low compared with the strong increase of EROD and PROD activities in MC-
Effect of oltipraz on rat hepatic CYPs

Fig. 3. PROD activities in liver microsomes from PB-treated rats in the presence or absence of OPZ. Microsomes were incubated with pentoxyresorufin in the absence or presence of OPZ (6.25, 12.5, 25, 50 and 100 µM) and PROD activity was measured. The values are expressed as mean ± SD (bars) of three different experiments.

Fig. 4. Effects of OPZ on EROD and PROD activities in primary cultures. Rat hepatocytes were cultured for 0–80 h in the absence or presence of 50 µM OPZ, and EROD and PROD activities were measured at different time intervals. Both activities are expressed as the ratio of OPZ-EROD activities decreased by 54.5 ± 2% and 49.7 ± 8%, respectively. In the case of PROD activities the values decreased by 70.0 ± 5% and 79.7 ± 1%, respectively. The percentage of inhibition was not markedly enhanced by increasing OPZ concentrations from 10 to 100 µM. A dose-dependent inhibition was also observed when liver microsomes prepared from PB-treated rats were incubated with OPZ (Figure 3). Similar effects were seen when OPZ was added to microsomes prepared from control and MC-, PB- and OPZ-treated hepatocytes in primary cultures (data not shown).

These results suggest that OPZ is firstly a reversible inhibitor of microsomal CYP1A1/2 and 2B1/2 and is lost during microsomal preparation, and at the same time an inducer of these enzymes. However, when compared with MC, OPZ appeared to be a weak inducer (Figure 1).

Effects of OPZ on CYP activities in primary rat hepatocyte cultures

Time course of inhibition by OPZ of EROD and PROD. As shown in Figure 4, OPZ given to parenchymal cells in primary culture inhibited EROD and PROD activities reversibly. A time-course analysis between 40 min and 72 h showed that inhibition by OPZ was already detectable after 40 min. Maximum inhibition was observed between 2–12 h of exposure when EROD and PROD activities fell to 16 and 38% of the controls by 24 h, the point at which medium and OPZ were renewed. OPZ added with medium renewal after 24 and 48 h resulted in further cycles of inhibition of activity followed by reversion towards control activities. By 72 h inhibition of EROD but not PROD appeared to have become progressive.

Effect of OPZ on MC and PB induction. Treatment of rat hepatocytes with OPZ together with the inducers MC (5 µM)
Fig. 5. Effect of OPZ on MC and PB-induced EROD and PROD activities. EROD and PROD activities were measured in primary cultures of rat hepatocytes after exposure to OPZ, MC or PB alone, or to OPZ plus MC or PB. The values are given as percentages of the values found in controls and results are expressed as mean ± SD (bars) of four experiments.

Table I. AFB1 biotransformation in rat hepatocytes in primary culture

<table>
<thead>
<tr>
<th>Treatment (48 h)</th>
<th>µM Unmetabolized AFB1</th>
<th>µM AFM1</th>
<th>µM AFBSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.5 ± 1.1</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>PB</td>
<td>1.92</td>
<td>0.2</td>
<td>1.1</td>
</tr>
<tr>
<td>MC</td>
<td>1.3 ± 0.2</td>
<td>1.7 ± 0.5</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>D3T</td>
<td>3.2 ± 0.9</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>OPZ</td>
<td>2.9 ± 0.2</td>
<td>0.02 ± 0.001</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>PB + OPZ</td>
<td>3.1</td>
<td>0.02</td>
<td>0.4</td>
</tr>
<tr>
<td>MC + OPZ</td>
<td>3.8</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Cells were treated by PB, MC, D3T or OPZ alone or OPZ plus PB or MC for 48 h. At 16 h after the last OPZ addition hepatocytes were incubated with 5 µM AFB1 during 8 h and the different metabolites were analyzed by HPLC. Results are expressed as the mean ± SD of three experiments or as the mean of two experiments.

Fig. 6. Metabolic rates of caffeine by rat hepatocyte cultures. Caffeine (CAF) 1 mM was incubated for 24 h with hepatocytes. Cultured hepatocytes were treated for 24 or 48 h with OPZ before caffeine treatment (OPZ then CAF) or 0, 24 or 48 h with OPZ and then with OPZ and caffeine simultaneously (OPZ + CAF) during 24 h. Results are the mean ± SD of three plates.

or PB (3.2 mM) for 48 h resulted in almost complete inhibition of induction of EROD and PROD, respectively (Figure 5).

Effects of OPZ on AFB1 metabolism. Since CYPs 1A and 2B are involved in AFB1 metabolism, we investigated the effect on it of OPZ, the inducers MC and PB and the combined effect of OPZ with MC or PB. The hepatocytes were preincubated with OPZ and the inducers for 48 h and then exposed to AFB1 for 8 h. The results in Table I show that OPZ inhibited AFB1 metabolism, whereas MC and PB induced it. MC caused an induction of the formation of AFM1 and AFBSG, whereas PB induced the formation of AFB1G. When hepatocytes were treated with OPZ in addition to MC or PB no induction occurred. D3T had a similar effect to OPZ.

Effects of OPZ on caffeine metabolism. A study of the effect of OPZ on caffeine metabolism by hepatocytes in culture gave similar results. Caffeine is mainly a substrate for CYP1A related enzymes and at the concentration used does not induce CYP1A enzyme (31). Its rate of metabolism resulted in a decrease of N-demethylation by 70% at 0 h and 50% at 24 h after pretreatment with OPZ (Figure 6). However when caffeine metabolism was studied for 24 h or more after OPZ treatment, no inhibition was observed.

Effects of OPZ on hepatic CYP expression and activities in the rat

The effect of OPZ on the metabolism of caffeine. To establish that CYP inhibition by OPZ is also effective in whole animals, a second series of experiments was carried out in which rats were given 25 mg/kg caffeine and 100 mg/kg OPZ by gavage, and serum caffeine metabolites were determined. OPZ was administered either 4 h before or simultaneously with caffeine. After a 8 h treatment, caffeine and its three major primary metabolites, TB, PX and TP, were measured in sera. Whereas in control animals the percentage of caffeine metabolized reached 58%, it did not exceed 6.7% in OPZ-treated animals. Moreover, OPZ administration changed the relative proportions of the three metabolites (Table II). The relative percentage of TP was increased while that of TB was decreased in animals treated with OPZ either simultaneously or at 4 h before caffeine administration. However, as was the case in vitro when caffeine was administered 24 h or more after OPZ treatment, no inhibition of metabolism was observed; on the contrary, metabolism was increased by 130–160% (Table II), the increase then declined 72 h after a 24-h OPZ treatment.

The effect of OPZ on mRNA for CYP associated with AFB1 metabolism. We investigated whether the increase of EROD and PROD activities in OPZ-treated rat liver had a pre-translational origin by analysis of mRNAs for CYP1A1, 2B1/2 and 2C11, the last named being a major CYP involved in AFB1 metabolism. An elevation was observed for CYP1A and CYP2B mRNAs (Table III), but no change in CYP2C11 mRNA levels was detected (data not shown). The increase of CYP1A and CYP2B mRNAs was observed as soon as 24 h after OPZ treatment; it was somewhat higher after 2 and 3 days, reaching 3- and 10-fold, respectively.
Effect of olitipraz on rat hepatic CYPs

Table II. Effects of OPZ treatment on caffeine metabolism

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metabolism (%)</th>
<th>Percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58.8 ± 18.7</td>
<td>100.0</td>
</tr>
<tr>
<td>OPZ + CAF</td>
<td>6.7 ± 2.2</td>
<td>11.3</td>
</tr>
<tr>
<td>OPZ 4 h</td>
<td>9.9 ± 6.4</td>
<td>16.9</td>
</tr>
<tr>
<td>OPZ 24 h</td>
<td>95.8 ± 4.2</td>
<td>162.8</td>
</tr>
<tr>
<td>OPZ 48 h (1)</td>
<td>77.3 ± 19.1</td>
<td>131.4</td>
</tr>
<tr>
<td>OPZ 48 h (2)</td>
<td>80.3 ± 17.4</td>
<td>136.4</td>
</tr>
<tr>
<td>OPZ 72 h (1)</td>
<td>74.9 ± 7.8</td>
<td>127.2</td>
</tr>
<tr>
<td>OPZ 72 h (3)</td>
<td>95.2 ± 0.8</td>
<td>161.8</td>
</tr>
</tbody>
</table>

Caffeine metabolism was quantified by HPLC in control and OPZ-treated rats. Animals were treated for 8 h with caffeine (CAF) and received OPZ either simultaneously or 4, 24, 48 and 72 h before. The number of gavages is indicated in parentheses. The results are expressed as mean ± SD of the values obtained with three animals.

Table III. Effects of OPZ on CYP1A and CYP2B1/2 mRNA expression

<table>
<thead>
<tr>
<th>CYP</th>
<th>Fold induction</th>
</tr>
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<tbody>
<tr>
<td>CYP1A</td>
<td>50.7 ± 2.4</td>
</tr>
<tr>
<td>CYP2B1/2</td>
<td>21.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>29.1 ± 5.4</td>
</tr>
</tbody>
</table>

Animals were maintained under control conditions or treated with 0.075% (w/w) of OPZ in the diet for 24, 48, 72 and 96 h. Total RNA was isolated and analyzed by Northern blot. Each value represents the fold induction of CYP1A or CYP2B1/2 mRNAs in OPZ-treated versus control rats measured by densitometry. Results are expressed as the mean of three experiments.

Discussion

OPZ has been shown to have chemoprotective activity against a number of carcinogens in a variety of rodent tissues, among which are liver, colon, skin, breast and trachea (32). In a recent study of OPZ using primary human hepatocytes, we demonstrated that it inhibited CYP1A2 and CYP3A4, the principal CYPs involved in AFB1 metabolism in man. This challenges an earlier assumption that OPZ acts only by inducing enzymes of carcinogen detoxication, especially GSTs (14). We show here that OPZ also inhibits CYP activities important for AFB1 metabolism in both rat hepatocytes in primary culture and the intact rat.

Several CYPs involved in AFB1 activation in the rat liver were investigated, namely CYP1A1, CYP2B1/2 and in addition CYP2C11 (33). OPZ was found to be a strong inhibitor of EROD and PROD activities, both in the livers of intact rats and in rat hepatocyte cultures. In normal rat liver, EROD activity is mainly supported by CYP1A1, whereas PROD activity appears to be supported by CYPs 2B1/2, 2C11, 2C6 and 3A2 (18). Since CYP2B1/2 is low, it may be suggested that OPZ inhibition of PROD activity reflected inhibition of CYPs other than CYP2B, particularly CYP2C11 and CYP3A2. However, the strong inhibition of PROD activity by OPZ in PB-treated microsomes and hepatocytes can be attributed to an inhibition of CYP2B1/2. Since inhibition affected several CYPs that were detectable after a 40-min exposure and peaked between 2 and 12 h, it would appear that OPZ, or a metabolite of OPZ, does not act indirectly by, for example, affecting CYP expression, but possibly by interacting with the heme moiety of the enzyme. The rapid reversible inhibition is presumably related to the short half-life of OPZ (34).

CYP inhibition was not observed in previous studies of the effect of OPZ on AFB1 metabolism when liver microsomes isolated from OPZ-treated rats were the object of study (35,36). As shown in the present work, inhibition due to OPZ does not survive the preparation of microsomes, presumably because enzyme bound OPZ dissociates and is lost.

Inhibition of CYP activities was largely relieved within 24 h. This indicates that OPZ does not behave as a suicide substrate in the rat, although it may do so in man where it appeared to be only partly reversible (14). The limited increase in inhibition of EROD and PROD activities from microsomes isolated from rats at 24 h or more after exposure to OPZ, is similar to that of other researchers who have used similar preparations (35).

As with human hepatocytes (14), in rat hepatocytes OPZ caused a marked decrease in total AFB1 metabolism. Our results and those of other researchers (33), using specific CYP inducers such as MC and PB, show that CYP1A1 and 2B1/2 are both able to metabolize AFB1 to AFBO. The dose of OPZ given is sufficient to inhibit the increased levels of CYPs that result from induction with MC and PB.

Oxidative biotransformation of caffeine in the rat to its three principal methylxanthine metabolites was also inhibited in a transient fashion by OPZ. N-1 demethylation (TB) due to CYP1A2 was inhibited more than N-7 demethylation (TP), which involves CYPs other than CYP1A1 (31), and it may be that N-7 demethylation known to be due to CYP(s) (5), as distinct from CYP1A1, is associated with CYP2B activity. The observation that the percentage of inhibition of PROD is less than that of EROD supports this view.

Following transient inhibition of CYP1A1 and 2B an induc-
AFB1 levels decline very slowly and have a toxic potential. However, as the inhibitory effect of OPZ declines and the metabolism of AFB1 is enhanced by OPZ-associated induction of activation, and provided the associated induction of detoxication is sufficient, successful chemoprotection is effected. Recently, Rao et al. (38) demonstrated that OPZ can also act as a chemoprotective agent against 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine(PhIP)-induced lymphoma. Since PhIP must be activated by CYP, the CYP inhibition by OPZ presented here can explain the inhibition of lymphoma as well as the induction of detoxication enzymes such as GSTs.

Although studies in the rat have proved to be a useful basis for studies in man, it has now been shown that chemoprotection by OPZ in man may be different for the following reasons: (i) there is no very effective GST for AFBO; and (ii) because of a genetic polymorphism 50% of the population lack the only effective GST namely GSTM1.

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