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Possible Isozyme-specific Effects of Experimental Malaria Infection with *Plasmodium berghei* on Cytochrome P450 Activity in Rat Liver Microsomes

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Abstract—We have investigated the effect of experimental malaria infection on rat cytochrome P450-mediated drug metabolism using ethoxyresorufin and metoprolol as probe compounds. Malaria infection caused a significant reduction in total intrinsic clearance of ethoxyresorufin in both low and high parasitaemia malaria compared with control (control 18·7±7·2; low parasitaemia 10·5±4·1; high parasitaemia 4·3±1·4 mL min^{-1}). However, clearance of metoprolol was unchanged in malaria infection compared with control (control 2·7±1·2; malaria 4·0±1·7 mL min^{-1}). The change in clearance of ethoxyresorufin was the result of a decrease in V_{max}, with no apparent change in K_m. There was no change in either V_{max} or K_m of metoprolol. These results indicate a possible isozyme-selective effect of experimental malaria.

Experimental malaria infection has been shown to alter hepatic drug metabolizing activity in animal models (Mansor et al 1990), and may show some selectivity of effect (Edwards et al 1992). As many clinically important phase 1 metabolic reactions are catalysed by isozymes of the P450 superfamily, it is essential to determine how these isozymes function in disease. The present study is part of an ongoing programme aimed at identifying those isozymes which may be affected by experimental malaria infection, using model substrates as experimental probes for specific isozymes of cytochrome P450. This investigation has assessed the activity of isozymes of rat cytochrome P450 using probe substrates ethoxyresorufin which represents principally cytochrome P4502C11 (CYP2C11) in non-induced rats (Nakajima et al 1990) and metoprolol which has been used as a marker for CYP2D1 (Smith 1991).

The rat was chosen as a model for malaria, since infection can easily be produced by inoculation of the rodent malaria parasite *Plasmodium berghei*. This model has been widely used (Mansor et al 1990, 1991a, b; Murdoch et al 1991) and allows accurate determination of parasitaemia, which may be correlated with relevant kinetic parameters such as intrinsic clearance.

Materials and Methods

Chemicals and reagents

Ethoxyresorufin, resorufin, bovine serum albumin (factor V), NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type XV), metoprolol and Folin and Ciocalteau's phenol reagent were obtained from the Sigma Chemical Company (Poole, UK). Acetonitrile (HPLC grade), methanol (HPLC grade), methyl-*tert*-butyl ether (HPLC grade), triethylamine, orthophosphoric acid, and 60% v/v perchloric acid (HPLC grade) were obtained from Fisons (Loughborough, UK). Guanoxan sulphate and α-hydroxymetoprolol were gifts from Dr M. S. Lennard (Department of Medicine and Pharmacology, University of Sheffield, UK).

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Animals

Young male Wistar rats (4 weeks old, approx. 90 g) were bred in the Departmental animal unit. For malaria infection studies, animals were infected with the rodent parasite *Plasmodium berghei*, as previously described (Kokwaro et al 1993). Rats with parasitaemia below 20% were placed into a low parasitaemia group, while those with parasitaemia above 20% were placed into a high parasitaemia group. Young animals were used as it is difficult to establish infection in older rats. The rats were killed by cervical dislocation seven days post-inoculation and the livers removed, quickly rinsed with cold phosphate buffer (0·07 M, pH 7·4), blotted dry, weighed and used to prepare microsomes according to the classical procedure of differential centrifugation (Lake 1987). The microsomes were suspended in phosphate buffer (0·075 M, pH 7·4, 1·5 mL) and stored at −80°C until use. Microsomal protein content was determined using bovine serum albumin as standard (Lowry et al 1951) and cytochrome P450 content was determined according to a standard spectrophotometric procedure (Omura & Sato 1964).

Incubation experiments

Preliminary experiments had shown that *O*-dealkylation of ethoxyresorufin to resorufin was linear for incubation times of 5–60 min, and quantities of protein ranging from 32 to 420 μg, following incubation with 5 μM ethoxyresorufin in an NADPH-generating system. The NADPH-generating system contained glucose-6-phosphate (10 μM), NADP (0·57 μM), $MgCl_2$ (10 mM) and glucose-6-phosphate dehydrogenase (2 units) dissolved in phosphate buffer (0·07 M, pH 7·4 2 mL). For kinetic studies, ethoxyresorufin (0·3–5 μM) was added to the NADPH-generating system dissolved in phosphate buffer (pH 7·4) and placed in a water bath (37°C). The contents were allowed to equilibrate for 2 min. The reaction

Table 1. *O*-De-ethylation of ethoxyresorufin by rat liver microsomes.

Experiment	n	Microsomal protein (mg (g liver)$^{-1}$)	Cytochrome P450 (ng (mg protein)$^{-1}$)	Parasitaemia (%)	K_m (μM)	V_{max} (nmol min^{-1} (nmol P450)$^{-1}$)	CL_{Tot} (min^{-1})
Control	5	6·4±1·4	0·60±0·11	—	0·80±0·16	0·43±0·08	18·7±7·2
Low malaria	5	5·6±0·5	0·52±0·01	16·6±3·0	1·15±0·36	0·31±0·06*	10·5±4·1
High malaria	6	3·6±0·3*	0·44±0·06*	31·8±5·5	1·25±0·40	0·28±0·09*	4·3±1·4

Mean±s.d. * $P<0·05$ compared with control.

was started by addition of microsomal protein (100 μg) and stopped after 15 min by immersion of the reaction tubes in an ice bath. The generated resorufin was determined as described below.

For the incubation studies with metoprolol, preliminary studies performed by incubating metoprolol (29·2 μM) showed that the production of α-hydroxymetoprolol was linear for microsomal protein amounts ranging from 0·05 to 1·6 mg and incubation times of 2–60 min. Therefore, for kinetic studies, metoprolol (0·8–34·2 μM) was added to an NADPH-generating system dissolved in phosphate buffer (0·2 M; pH 7·4; 0·2 mL) and placed in a water bath at 37°C. The NADPH-generating system comprised glucose-6-phosphate (4 μM), NADP (0·4 μM), $MgCl_2$ (10 mM) and glucose-6-phosphate dehydrogenase (0·4 units). Following equilibration (2 min) the reaction was started by addition of microsomal protein (0·4 mg) and stopped after 20 min by addition of perchloric acid (50 μL, 60% solution). α-Hydroxymetoprolol was determined as described below.

Determination of resorufin and α-hydroxymetoprolol

The amount of resorufin formed was determined according to the spectrofluorimetric method of Burke & Mayer (1974). The fluorimeter was calibrated daily with resorufin (15 μL 50 μM stock in ethanol).

α-Hydroxymetoprolol was determined using the HPLC method of Lancaster et al (1990) with the following modifications. To each polypropylene vial containing 50 μL 60% perchloric acid and 450 μL of the incubation mixture was added 1200 ng internal standard (guanoxan sulphate dissolved in 1·15% KCl). This was vortexed (1 min) then centrifuged (600 *g*, 5 min), and the supernatant was transferred into a clean tube containing NaOH (4 M 500 μL). Methyl-*tert*-butyl ether (5 mL) was added and the mixture vortexed (1 min) then centrifuged (600 *g*, 5 min). The organic layer was removed and evaporated to dryness in a water bath (40°C) under nitrogen. The residue was reconstituted in mobile phase (100 μL; water 91: acetonitrile 9, containing 1% triethylamine adjusted to pH 3·0 with orthophosphoric acid). The HPLC system used comprised a SpectraPhysics P100 isocratic pump, a Rheodyne 7125 valve injector fitted with a 100 μL loop, a 5 μ ODS 2 Spherisorb column (10×4·6 mm; Capital HPLC, Bathgate, UK), a Pye Unicam LC871 UV-vis detector (set at 225 nm and attenuation of 0·02 aufs) and a SpectraPhysics SP4100 computing integrator. Mobile phase flow rate was 1 mL min^{-1} at ambient temperature. Concentrations of α-hydroxymetoprolol were determined from peak area ratios with reference to the standard curve.

Calculation of kinetic parameters

The rate of formation of resorufin and α-hydroxymetoprolol from their respective substrates was calculated as nmol formed (mg microsomal protein)$^{-1}$ min^{-1}. The untransformed rate data were fitted to the Michaelis–Menten equation for a single enzyme reaction, confirmed by consistently linear Hanes-Woolf plots using the iterative nonlinear regression program GRAFIT (Leatherbarrow 1990) according to the following model:

$$V = V_{max} \cdot S/K_m + S \quad (1)$$

where K_m = affinity of the enzyme for the substrate and V_{max} = capacity of the enzyme.

Under first-order conditions equation 1 can be written:

$$V/S = V_{max}/K_m = CL \quad (2)$$

where V = formation rate of resorufin or α-hydroxymetoprolol, S = ethoxyresorufin or metoprolol concentration and CL = intrinsic clearance. Intrinsic clearance is a measure of the activity of drug metabolizing enzymes (Rane et al 1977).

The intrinsic clearance by the whole liver, CL_{Tot}, was calculated as the ratio of V_{max}, expressed per nmol cytochrome P450, and K_m, multiplied by the total amount of cytochrome P450 in the liver according to the following equation (Buters & Reichen 1990):

$$CL_{Tot} = (V_{max}/K_m) \cdot \text{cytochrome P450} \quad (3)$$

Statistical analysis of estimated parameters

The non-parametric Wilcoxon rank sum test was used for the comparison of the means of parameters from two groups. $P<0·05$ was taken as significant.

Results

Drug analysis and variability

Using initially drug-free microsomal suspensions with added

Table 2. α-Hydroxylation of metoprolol by rat liver microsomes.

Experiment	n	Microsomal protein (mg (g liver)$^{-1}$)	Cytochrome P450 (ng (mg protein)$^{-1}$)	Parasitaemia (%)	K_m (μM)	V_{max} (nmol min^{-1} (nmol P450)$^{-1}$)	CL_{Tot} (min^{-1})
Control	5	5·1±0·1	0·50±0·16	—	12·4±3·9	1·19±0·26	2·7±1·2
Malaria	4	4·9±1·0	0·45±0·06	26·5±5·5	16·6±6·6	1·02±0·05	4·0±1·7

Mean±s.d. No significant differences were found compared with control.

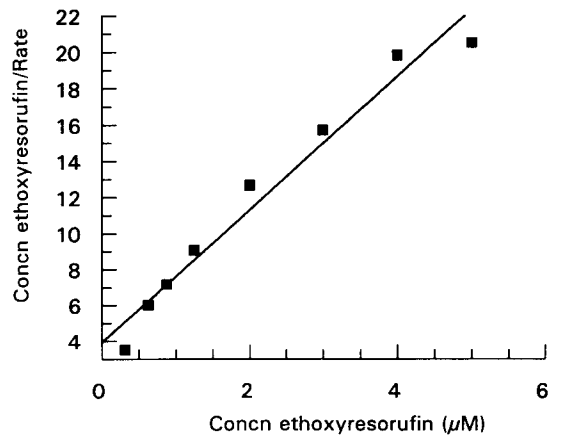

FIG. 1. Hanes-Woolf plot for ethoxyresorufin *O*-de-ethylation by rat liver microsomes in-vitro.

known amounts of α-hydroxymetoprolol (80 and 225 μg L^{-1}) the values determined were 74±4 and 220±2 μg L^{-1} (n=4), respectively. Coefficients of variation for the spectrofluorometric determination of resorufin were less than 10%.

Physiological and biochemical indices (Tables 1, 2)
Microsomal protein content (mg (g liver)$^{-1}$) was not significantly different in malaria infection compared with controls for the metoprolol studies or in the low parasitaemia malaria compared with control for ethoxyresorufin studies, yet in the high parasitaemic group, it was much reduced. Previous investigations (Kokwaro et al 1993) have indicated there to be no consistent relationship between microsomal protein and parasitaemia.

In ethoxyresorufin studies, total cytochrome P450 (nmol (mg microsomal protein)$^{-1}$) was numerically reduced in both low and high parasitaemia malaria, but only the high parasitaemia showed statistical difference. In metoprolol studies, total cytochrome P450 was not reduced significantly by malaria compared with the control.

Malaria and ethoxyresorufin O-de-ethylation
Kinetic parameters and a Hanes-Woolf plot suggesting the involvement of a single enzyme in ethoxyresorufin *O*-de-ethylation are shown in Table 1 and Fig. 1, respectively. Although there was no significant change in K_m compared with controls, after correction for estimated microsomal protein and cytochrome P450, V_{max} was reduced significantly in both low and high parasitaemia malaria. Consequently, total intrinsic clearance of ethoxyresorufin was markedly reduced.

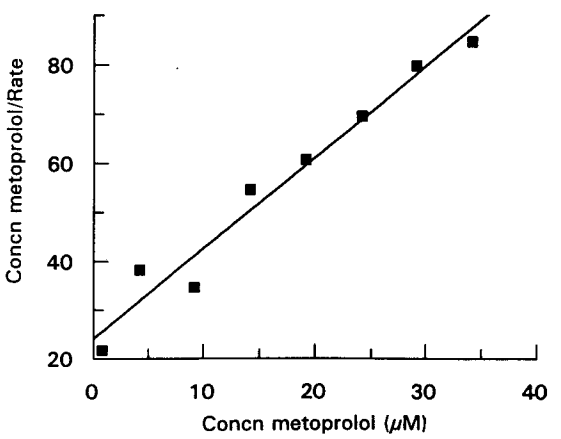

FIG. 2. Hanes-Woolf plot for metoprolol α-hydroxylation by rat liver microsomes in-vitro.

Malaria and metoprolol α-hydroxylation
Kinetic parameters and a Hanes-Woolf plot suggesting the involvement of a single enzyme in metoprolol α-hydroxylation in malaria infection are shown in Table 2 and Fig. 2, respectively. Neither K_m nor corrected V_{max} was affected by malaria infection.

Discussion

It has been reported previously that malaria infection depresses phase I and phase II drug metabolism (McCarthy et al 1970; Mansor et al 1990, 1991a, b). The mechanisms accounting for these observations have not fully been elucidated, but several explanations have been suggested based upon known pathophysiological changes which occur during malaria infection. These include alterations in hepatic structure and decreased cytochrome P450 (Tekwani et al 1988) and direct impairment of hepatic P450-dependent mono-oxygenase activity by *P. berghei* (Alvares et al 1984). While a reduction in hepatic cytochrome P450 may account partially for the decrease in CL_{Tot} for ethoxyresorufin, it cannot explain the relatively selective effect of malaria infection, since a decrease in cytochrome P450 by 14% produced a significant decrease in CL_{Tot} of ethoxyresorufin, while a reduction of similar magnitude had little effect on CL_{Tot} of metoprolol. This apparently selective effect of malaria infection may be due to differences in the influence of the infection on the isozymes involved in the metabolism of these compounds in the rat.

Such a selective effect may relate, in part, to elevated levels of cytokines in malaria-infected rats. Elevated levels of tumour necrosis factor α, and interleukin-6 are known to be a clinical feature of malaria infection (Grau et al 1989), and cytokines have been reported to be associated with depressed microsomal drug metabolism in-vivo and in-vitro (Ghezzi et al 1986; Chen et al 1992). Differential effects of cytokines on cytochrome P450 isozymes in rat liver have been reported (Ferrari et al 1992, 1993) and our findings with ethoxyresorufin and metoprolol may, in part, reflect the differential effects of cytokines on the metabolism of the two probes. However, interleukin-1, a major inflammatory mediator in-vivo has been reported to depress both ethoxyresorufin *O*-de-ethylation in rat hepatocytes (Ferrari et al 1992) and debrisoquine and bufuralol mono-oxygenases in rat liver microsomes (Kurokohchi et al 1992).

Another factor which may contribute to the depression of drug metabolism in livers of infected rats is the malaria pigment haemozoin. The presence of haemozoin in hepatocytes has been reported to be associated with decreased cytochrome P450 activity in experimental animals (Muller-Eberhard et al 1983). However, accumulation of haemozoin alone cannot wholly explain the differential effect of the malaria on metabolism of ethoxyresorufin and metoprolol.

Since reduction in CL_{Tot} for ethoxyresorufin appears to be due to a decrease in the capacity of the enzyme (Fig. 1), we suggest that malaria infection causes an alteration in capacity but not affinity of the isozyme involved in the metabolism of ethoxyresorufin. While a reduction in V_{max} may suggest a reduction in the amount of enzyme present, it is not known if malaria infection alters the expression of cytochrome P450 in rat liver. Whatever the mechanism, changes in expression of specific P450 isozymes may produce alterations in the hepatic disposition of drugs used in the treatment of malaria and this merits further investigation.

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