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The effect of malaria infection on antipyrine metabolite formation in the rat

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Antipyrine is a model compound commonly used to study the influence of environmental, genetic factors, disease states and other drugs on hepatic drug metabolizing enzyme activity *in vivo* [1]. Antipyrine is a suitable model substrate for studies of drug oxidation [2] since its absorption after oral dose is 100% and there is no presystemic elimination. In addition, its elimination is almost entirely by metabolism, and protein binding is minimal. It distributes rapidly into total body water [3] and is a low clearance drug with its elimination being independent of hepatic blood flow [1]. Present knowledge has demonstrated unequivocally that there are multiple forms of cytochrome P450 and studies with animals and man suggest that as many as 20 or more different P450 genes may be expressed in the body at one time [4, 5]. With this in mind the antipyrine test recently has been improved to assess the antipyrine metabolite profiles since the formation of its major metabolites is reported to be regulated by different forms of cytochrome P450 [6–9]. These modifications allow the examination of independent drug metabolizing pathways catalyzed by different drug-oxidizing enzymes in one test.

Malaria infection (MI) has been reported to cause perturbations in hepatic enzymes activities of the host [10–12]. With respect to hepatic drug metabolizing enzyme systems, both phase 1 biotransformation processes and phase 2 conjugation reactions are suppressed during MI in rodents [12, 13, 14]. However there is little information concerning the selectivity of the effect of MI on drug metabolizing enzymes. Knowledge in this area will be important since antimalarial drugs are eliminated extensively by hepatic metabolism and are generally low to intermediate clearance drugs many of which have narrow therapeutic indices. Therefore it might be expected that their disposition will be affected by changes in the hepatic enzyme activity produced by MI. In order to gain some insight regarding this issue, we have studied the effect of MI on antipyrine metabolism in the rat and compared these data with those obtained pre-infection and during convalescence after the infection had been cured with halofantrine.

Materials and Methods

Antipyrine was purchased from BDH (Poole, U.K.). 4-Hydroxyantipyrine and norantipyrine were a gift from Dr D. McKillip (ICI, Alderley Edge, U.K.) and 3-hydroxymethylantipyrine was a gift from Professor D. Breimer (University of Leiden, The Netherlands).

Malaria model (rat). Male T.H.W. mice (25–30 g) were infected with the rodent specific malaria parasite *Plasmodium berghei* RC-chloroquine resistant (ED_{50} > 150 mg/kg) strain using an inoculation (i.p.) of 10^6 parasitized erythrocytes from previously infected animals. Blood samples were removed from the tail vein for daily monitoring the degree of parasitaemia by Giemsa (BDH, Poole, U.K.) stained thin blood films evaluated under a light microscope. When the degree of parasitaemia exceeded 25%, blood was collected from the brachial artery of the anaesthetized mice and transferred into lithium heparin tube (L.I.P. Ltd, Shipley, U.K.).

Study design. Young (5–8 weeks) male Sprague–Dawley rats (70–120 g, $N = 6$) were infected with the RC strain of *P. berghei* by i.p. injection of parasitized red blood cells (0.01 mL; 10^6) from infected mice. Parasitaemia as assessed by observation of a Giemsa stained thin blood film was allowed to develop to a level of 25–30% prior to treatment with halofantrine, a drug with proven efficacy against chloroquine resistant parasites [15]. Control animals (N = 6) were non-infected and matched for age, weight,and sex. Rats were placed individually in metabolism cages and supplied with water but no food for the evening prior to the study. Urine was collected 0–24 hr and sodium metabisulphite (Fisons, Loughborough, U.K.; 1.0 mg/mL) was added to prevent decomposition of antipyrine metabolites. Samples were stored at −20° until analysis.

Drug administration. Antipyrine (15 mg/kg orally) was administered on days 1, 7, 14, 18, 25, 32 and 48. Infection was initiated when the *P. berghei* suspension was injected into rats in the test group on day 8. Halofantrine hydrochloride (SK&F, Welwyn, U.K.; 8 mg/kg in sunflower oil i.p.) was administered on day 15 to all animals.

Determination of microsomal protein and cytochrome P450. At the end of the experiment (day 49) rats were killed by cervical dislocation and livers excised for determination of microsomal protein [16] and cytochrome P450 content [17]).

Table 1. Excretion of antipyrine metabolites as percentage of dose in two groups of rats

	Before malaria		During malaria		After malaria							
	(pooled data day 1 & day 7)		(day 14)		(day 18)		(day 25)		(day 32)		(day 48)	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
AP	1.65	1.66	1.7	8.1*	1.3	1.7†	0.69	0.63	—	—	—	—
	(0.3)	(0.3)	(0.4)	(1.1)	(0.3)	(0.2)	(0.31)	(0.30)				
NORA	16.9	16.7	17.1	17.3	17.9	17.9	18.5	18.3	18.7	18.3	18.8	18.8
	(1.4)	(1.4)	(0.9)	(0.7)	(0.7)	(0.8)	(0.6)	(0.7)	(0.4)	(0.6)	(0.6)	(0.7)
HMA	23.3	24.3	24.5	21.4*	25.2	24.1†	26.2	25.1	27.0	26.0	27.8	27.2
	(1.5)	(1.3)	(1.2)	(0.7)	(0.9)	(0.6)	(0.8)	(1.1)	(0.7)	(1.1)	(0.9)	(1.0)
OHA	19.8	19.8	20.1	15.5*	21.1	20.1	22.1	20.9	22.7	21.9	23.9	22.9
	(1.3)	(1.2)	(0.9)	(1.3)	(0.8)	(0.9)	(1.1)	(0.9)	(1.0)	(1.1)	(0.8)	(1.2)

Results are mean ± SD.
* $P < 0.001$; † $P < 0.05$ compared with control.
The following abbreviations are used: antipyrine (AP); norantipyrine (NORA); 4-hydroxyantipyrine (OHA) and 3-hydroxymethylantipyrine (HMA).

Assay of antipyrine and its metabolites. Urinary antipyrine and its metabolites were measured by the HPLC method as described [18] with some slight modifications. Chromatographic separation of antipyrine and its metabolites, and phenacetin (internal standard) was carried out on a μBondapak C18 "Rad-Pak" column housed in a Z-module fitted with a CN-Guard Pak (Waters, Millipore, Harrow, U.K.). The mobile phase consisted of methanol: phosphate buffer [0.05 M; pH 6.8; 40:60 (v/v)] flowing at 3 mL/min. Urinary extraction procedures were as described by Danhof *et al.* [18] with additional precautions as described by Teunissen *et al.* [19] to enhance the stability of Phase 1 metabolites during sample preparation. Conjugated metabolites were hydrolysed [20] with β-glucuronidase/sulphatase (Helix pomatia, Type H-1, 184 units/mL urine; Sigma Chemical Co., Poole, U.K.).

Data analysis. Data are presented as mean ± SD. Coefficients of variation (assay precision) were obtained from the ratio of the standard deviation to the mean. Multiple means were analysed using 1-way analysis of variance with comparisons between individual groups assessed by multiple range tests.

Results

Chromatography. The coefficients of variation for the determination of the individual metabolites were as follows; antipyrine (AP) 6% at 5 μg/mL; 4-hydroxyantipyrine (OHA) 5% at 40 μg/mL; 3-hydroxymethylantipyrine (HMA) 4.4% at 20 μg/mL and norantipyrine (NORA) 8.3% at 20 μg/mL. At these concentrations, the recovery from blank rat urine of AP and its metabolites, OHA, HMA and NORA were 95 ± 3% (N = 5), 90 ± 2% (N = 5), 55 ± 4% (N = 5) and 87 ± 3% (N = 5), respectively. The minimum detectable concentrations for AP, OHA, HMA and NORA were 150, 200, 400 and 250 ng/mL, respectively, which provides sufficient sensitivity for these studies.

Malaria and antipyrine biotransformation. The degree of parasitaemia established in the rats at 3 and 5 days post-inoculation was observed to be reproducible. At these time points the degree of parasitaemia was found to be 12.8 ± 1.7 and 28.2 ± 2.2%, repectively.

MI resulted in a significant increase in urinary unchanged AP and a significant decrease in the recovery of MA and OHA respectively. No significant change was observed in the recovery of NORA in infected rats as compared to controls (Table 1). Treatment with halofantrine, produced a complete cure of MI. After treatment with halofantrine a relatively trivial but statistically significant increase in unchanged AP was observed when compared to control and only the formation of HMA was affected in the group of infected rats (Table 1.) There was a significant decrease in the percentage of unchanged AP recovered over the study periods in both control ($P < 0.001$) and infected ($P < 0.001$) rats. AP was not detected in urine of either group by day 32 and 48 (Table 1). There was no significant difference in hepatic microsomal protein (control: 18.0 ± 2.3 vs test: 17.5 ± 1.4 mg/g liver) or cytochrome P450 (control: 0.94 ± 0.10 vs test: 0.88 ± 0.07 nmol P450/mg protein) between groups at the end of the experiment.

Discussion

Antipyrine is oxidized by different forms or populations of cytochrome P450 to four primary metabolites in rat [9]. All metabolites are excreted in urine as conjugates [21]. In rat, the major conjugation route is sulphation while glucuronidation is the main conjugation reaction in man [22]. This probe drug has been used successfully in metabolic studies in rats and man to assess common pathways in metabolic oxidation [23, 24]. In rats, the formation of HMA is associated with the phenobarbitone (PB) inducible enzymes while OHA is associated with the methylcholanthrene (MC) type of cytochrome P450 and NORA may also be formed by the MC-inducible form of cytochrome P450 [6–9]. Our studies suggest that malaria infection can selectively reduce the formation of both HMA and OHA but not NORA. The total recovery of the administered dose is about 62–70% in both groups which highlights the importance of aromatic ring hydroxylation to form 4′,4′-dihydroxyantipyrine (DHA) which represents about 11–18% [19, 25] of the administered dose in rat but only 3–6% in man [25]. This demonstrates the relevance of measuring DHA to establish an overall picture of AP metabolism in the rat which was not possible in the present study due to DHA being unavailable. Our results suggest however that DHA formation might not be affected by MI, since the total amount of dose recovered in control and infected rats is not significantly different (Table 1).

Following treatment, the formation of HMA (which appears to be associated with the PB type of cytochrome P450) remains reduced and return to normal only after 9 days following treatment with halofantrine. In contrast, the formation of OHA apparently has a shorter recovery time of 3 days. At the end of the experiment (49 days), the hepatic enzyme activities as determined by measurement of hepatic microsomal protein and cytochrome P450 are not significantly different in either group of rats indicating that treatment of the disease can restore the function of the hepatic microsomal mixed-function system. It is well documented that MI decreases hepatic cytochrome P450 and associated monoxygenase activity in *P. berghei* infection [11, 12, 26]. AP is not detectable in urine by day 32 suggesting autoinduction over the period of this investigation (Table 1). Similar observations have been reported in man [27] and previously in the rat, although at a much higher dose [28].

In summary, we have shown that malaria infection can impair selectively the formation of antipyrine metabolites in the rat. During malaria, a significant increased urinary levels of unchanged antipyrine was observed (control: 1.7 ± 0.4 vs test: 8.1 ± 1.1% of dose, $P < 0.001$). This was associated with significantly decreased excretion of 3-hydroxymethylantipyrine (control: 24.5 ± 1.2 vs test: 21.4 ± 0.7%, $P < 0.001$) and 4-hydroxyantipyrine (control: 20.1 ± 0.9 vs test: 15.5 ± 1.3%, $P < 0.001$) but not norantipyrine compared to control. Following treatment of the malaria infection with halofantrine, only the formation of 3-hydroxymethylantipyrine (control: 25.2 ± 0.9 vs test: 24.1 ± 0.6%, $P < 0.05$) is impaired. The implications of these findings in relation to metabolism of other antimalarial drugs during malaria remains to be elucidated. Further work is needed to determine the changes in the pharmacokinetics of AP and its metabolites before, during and after MI in the rat in order to give a better insight into the effect of MI on hepatic drug metabolism.

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