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Non-induction of extrahepatic antipyrine and metronidazole metabolism evaluated from partially hepatectomized rats

I. B. JEDING†, S. LOFT and H. E. POULSEN

Department of Pharmacology, University of Copenhagen, Denmark

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1. The effect of β-naphthoflavone (BNF), given i.p. ($n=9$) and orally ($n=9$), on the metabolism of antipyrine and metronidazole was investigated in rats.

2. The clearances of antipyrine and metronidazole were determined on a single saliva sample. The rates of formation of antipyrine and metronidazole metabolites were determined from a 20 h urine sample and saliva clearance.

3. Administration of β-naphthoflavone i.p. was significantly more effective than oral dosage on the induction of antipyrine and metronidazole metabolism ($p<0.05$).

4. The capacity of extrahepatic tissues to metabolize antipyrine and metronidazole was quantitatively assessed in rats with and without pretreatment with β-naphthoflavone immediately after sham operation or 70% partial hepatectomy ($n=40$).

5. Antipyrine and metronidazole clearances correlated with liver weight in induced and non-induced rats. Linear regression of antipyrine and metronidazole clearances did show a non-significant Y-intercept ($p>0.05$), indicating a negligible extrahepatic metabolism in both induced and in non-induced rats.

6. From a quantitative point of view this study indicates that induction of extrahepatic cytochrome P450 metabolism of antipyrine and metronidazole is negligible.

Introduction

Antipyrine and metronidazole are useful for the study of cytochrome P450 xenobiotic-metabolizing enzymes in many species. Treatment with polycyclic aromatic hydrocarbons (PAH) *in vivo* increases the *in vivo* metabolism of antipyrine significantly in both man and rat (Rhodes and Houston 1983, Loft *et al.* 1991), whereas the inductive effect of the PAH-type inducer 3-methylcholanthrene measured *in vitro* in preparations of isolated rat hepatocytes (Loft and Poulsen 1989) and microsomal fractions of rat liver (Kahn *et al.* 1982) is minimal. This possibly indicates the induction of extrahepatic antipyrine metabolism.

The distribution of cytochrome P450 is extrahepatic as well as hepatic, and 450 induction also occurs in extrahepatic tissues in virtually all mammalian tissue studied. Extrahepatic induction is especially prominent with PAH-type inducers (Okey 1990).

The clearance of antipyrine correlated closely with liver weight in non-induced rats with graded hepatectomy up to 90% (Hansen and Poulsen 1986, Pilsgaard and Poulsen 1984, Poulsen 1985). That the regression intercept approximates to zero is taken as evidence that antipyrine is almost exclusively metabolized in the liver. By the same token, we investigated the possibility that β-naphthoflavone is associated with induction of extrahepatic metabolism of antipyrine and metronidazole, by

†Address correspondence to: Inge B. Jeding, Department of Pharmacology, Panum Institute, Blegdamsvej 3 (18/6), DK-2200 Copenhagen N, Denmark.

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comparing metabolism in partially hepatectomized and sham-operated rats. The β-naphthoflavone induction of intestinal metabolism of the two compounds was also investigated by comparing the oral and intraperitoneal administration of β-naphthoflavone.

Experimental

Chemicals

Antipyrine and metronidazole, thiopentone and pilocarpine were obtained from the Danish Hospital Pharmacies, Leo Ltd and DAK (Copenhagen, Denmark) respectively. β-Naphthoflavone was obtained from Aldrich (Steinheim, Germany) and used in solution with peanut oil (DAK Copenhagen, Denmark). Glucuronidase/arylsulphatase (Gluculase® Type H-3 from *Helix pomatia*) were obtained from Sigma. All solvents were of analytical or chromatographic grade.

Animals

Laboratory-bred male Wistar rats (210–230 g) were housed under constant temperature and humidity in 12-h light cycle with free access to food (Altromin®) and tap water. When housed in metabolic cages the rats had free access to ground Altromin and 2% sucrose water.

Experimental design

For the study comparing the administration of β-naphthoflavone orally with i.p. administration, 36 rats were divided into four groups of nine rats. Two groups were pretreated with β-naphthoflavone in peanut oil (60 mg/kg body weight) orally or i.p., respectively, for 3 days. The other two groups were given peanut oil orally or i.p., respectively, for 3 days. A mixture of antipyrine and metronidazole was given i.p., on the fourth day as described below. Antipyrine and metronidzole have no influence on the metabolism of each other when given in a mixture (Loft *et al.* 1991).

For the study of extrahepatic metabolism, 40 rats were divided into four groups. Two groups of 12 rats were pretreated with β-naphthoflavone i.p. (60 mg/kg body weight) for 3 days and the other two groups of eight rats were treated with peanut oil i.p. One group of 12 rats pretreated with β-naphthoflavone and one group of eight rats treated with peanut oil (controls) underwent 70% partial hepatectomy, as described by Higgens and Anderson (1931), while two similarly treated groups of 12 or eight rats were sham operated. During surgery all rats were anaesthetized with diethyl ether. Sham operation consisted of exteriorization, manipulation and replacement of the liver.

Upon recovery from anesthesia the rats received a mixed solution of antipyrine (14 mg/kg body weight) and metronidazole (11·5 mg/kg body weight) by the i.p. route. Immediately after administration the rats were placed separately in metabolic cages and urine was collected for 20 h.

From each rat a single sample of saliva was collected, from the submandibular crevice, in capillary tubes. The optimal time interval from antipyrine/metronidazole administration to the collection of the one saliva sample for determination of the saliva clearances was estimated according to Døssing *et al.* (1982, 1983, and Loft *et al.* 1988, Pilsgaard and Poulsen 1984). The sampling time was calculated to be 3 h for non-induced sham-operated rats, and 6 h for non-induced partially hepatectomized rats. For induced rats the time interval was calculated to be 1·5 and 2·5 h, for sham-operated and partially hepatectomized rats, respectively.

To obtain a suitable amount of saliva (300 μl) for clearance determination the salivary flow rate was stimulated by subcutaneous administration of 1·5 mg/kg body weight of pilocarpine. Pilocarpine in the dose used for saliva stimulation has been shown not to alter the salivary half-life of antipyrine, or to change the concentration ratio between plasma and saliva (Welch *et al.* 1975). The same was assumed for metronidazole (Loft *et al.* 1991).

The one-sample clearances of antipyrine and metronidazole were calculated as described elsewhere (Pilsgaard and Poulsen 1984). The fractional clearance of metabolites, representing each elimination pathway, was calculated as the product of the clearance and the fraction of the dose excreted as that particular metabolite.

Two rats were excluded from the study, one which died at hepatectomy and one which suffered liver ischaemia after sham operation. Therefore the final number of rats comprised two non-induced groups with eight rats each, and two induced groups with 11 rats each.

Analytical procedures—saliva and urine

Analysis of antipyrine and metronidazole in saliva were carried out by high performance liquid chromatography (h.p.l.c.) monitored with a u.v.-detector according to Loft *et al.* (1991). Urine samples were assayed in duplicate for parent compounds and metabolites, also described previously by Loft *et al.* (1991). There is no interference between antipyrine, metronidazole and their metabolites with these assays (Loft *et al.* 1991).

Statistics

The number of rats in each treated group was based on knowledge of expected variation in drug analysis, especially with respect of the increase after β-naphthoflavone induction (Loft *et al.* (1991). Regression and correlation analyses were done by the method of least-squares. Clearance, fractional clearance of the metabolites, and urinary recovery were compared between groups by means of one-way analysis of variance. All clearance data were logarithmically transformed to obtain homogeneity of variance. Estimates of the regression coefficients were investigated by a paired *t*-test. The level of statistical significance was set at $p<0.05$.

Results

Effect of route of β-naphthoflavone administration

Antipyrine. Pretreatment of rats with β-naphthoflavone i.p. increased the clearance of antipyrine three-fold, i.e. significantly more than oral administration, which doubled the clearance ($p<0.05$, Table 1A). When β-naphthoflavone was administered i.p. the fractional clearance to norantipyrine (NORAP) and 4-hydroxyantipyrine (OHAP) increased seven- and six-fold, respectively ($p<0.05$), compared with controls. After oral β-naphthoflavone pretreatment the increases were four- and 2·5-fold, respectively ($p<0.05$), compared with controls. The fractional clearance to 3-hydroxymethylantipyrine (HMAP) was identical to controls irrespective of the route of β-naphthoflavone administration. The renal clearance of unchanged antipyrine was unaltered between groups.

The urinary recovery of antipyrine and metabolites was 69 ± 21% (mean of group ± SD) and 62 ± 17% in the i.p and oral non-induced groups, respectively, and 59 ± 16 and 60 ± 22% of the total dose in the i.p. and oral β-naphthoflavone-treated groups, respectively.

Table 1. Influence of routes of β-naphthoflavone administration on (A) antipyrine and (B) metronidazole metabolism in rats.

A	Total clearance of antipyrine	Clearance to HMAP	Clearance to NORAP	Clearance to OHAP	Renal clearance	Recovery (% of dose)
Oil; oral	5·42 ± 1·36	1·84 ± 0·66	0·37 ± 0·14	0·70 ± 0·27	0·36 ± 0·20	62 ± 17
Oil; i.p.	5·88 ± 0·77	2·11 ± 0·56	0·48 ± 0·18	0·82 ± 0·29	0·62 ± 0·33	69 ± 21
BNF; oral	10·01 ± 2·64†	2·23 ± 0·76	1·50 ± 0·98†	1·75 ± 0·70†	0·60 ± 0·54	60 ± 22
BNF; i.p.	17·11 ± 4·65†‡	1·67 ± 0·70	3·31 ± 1·76†‡	4·63 ± 2·00†‡	0·59 ± 0·47	59 ± 16
B	**Total clearance of metronidazole**	**Clearance to MAA**	**Clearance to HM**	**Clearance to GM**	**Renal clearance**	**Recovery (% of dose)**
Oral; oil	6·74 ± 1·81	0·37 ± 0·20	0·18 ± 0·10	0·61 ± 0·20	0·90 ± 0·49	34 ± 9
Oil; i.p.	6·10 ± 1·31	0·31 ± 0·14	0·16 ± 0·07	0·65 ± 0·39	1·04 ± 0·28	36 ± 7
BNF; oral	7·79 ± 2·81	0·60 ± 0·23	0·39 ± 0·10	0·77 ± 0·36	0·91 ± 0·16	38 ± 12
BNF; i.p.	7·99 ± 1·70	1·46 ± 0·80†‡	1·07 ± 0·52†‡	0·89 ± 0·35	1·01 ± 0·49	54 ± 15

Control rats and rats pretreated with BNF (60 mg/kg body weight) received antipyrine i.p. (14 mg/kg body weight) and metronidazole i.p. (11·5 mg/kg body weight). The total clearance and fractional clearances were determined from one saliva sample and urine collected for 20 h, respectively.

All values of clearance are termed as ml/min per kg body weight and are means ± SD; $n=9$.

† $p<0.05$ *versus* control with the same route of administration.

‡ $p<0.05$ *versus* oral administration of BNF.

BNF, β-naphthoflavone in solution with peanut oil; oil, peanut oil; oral, oral administration; i.p., intraperitoneal; HMAP, 3-hydroxymethylantipyrine; NORAP, norantipyrine; OHAP, 4-hydroxyantipyrine; MAA, metronidazole acetic acid; HM, hydroxymetronidazole; GM, metronidazole glucuronide; renal, clearance by renal excretion of unchanged compound.

Metronidazole. The saliva clearance of metronidazole was not significantly different after oral and i.p. β-naphthoflavone administration (table 1 B). The fractional clearance to the two metabolites, metronidazole acetic acid (MAA) and hydroxymetronidazole (HM), were increased after pretreatment with β-naphthoflavone, both when administration was i.p. and oral. The MAA rate of formation was increased five-fold after i.p. administration, i.e. significantly more than the two-fold increase after oral administration ($p<0.05$). The clearance to HM was increased seven-fold after i.p., and two-fold after oral administration, respectively ($p<0.05$). Clearance by glucuronidation was not significantly changed, neither was the renal clearance of unchanged metronidazole.

The urinary recovery of metronidazole and metabolites was $36\pm7\%$ (mean of group $\pm$ SD) and $34\pm9\%$ in the i.p. and oral non-induced groups, respectively, and 54 ± 15 and $38\pm12\%$ of the total dose in the i.p. and oral β-naphthoflavone-treated groups, respectively.

Effect of partial hepatectomy on the metabolism of antipyrine and metronidazole

In sham-operated rats with or without β-naphthoflavone pretreatment, the clearance of antipyrine and metronidazole and the metabolite profiles (table 2) were similar to those of the i.p.-treated rats from the first part of the study concerning the route of β-naphthoflavone administration (table 1).

Antipyrine. In both β-naphthoflavone-pretreated and non-induced rats the 70% partial hepatectomy decreased the clearance of antipyrine and the metabolite formation rates to a similar degree (table 2 A). There were significant correlations

Table 2. Influence of β-naphthoflavone induction and 70% partial hepatectomy on (A) antipyrine and (B) metronidazole metabolism in rats.

A	Total clearance of antipyrine	Clearance to HMAP	Clearance to NORAP	Clearance to OHAP	Renal clearance	Recovery (% of dose)
Control; SO	4·82±1·09	1·82±1·03	0·60±0·39	0·76±0·39	0·32±0·23	70±23
Control H	1·88±0·45†	0·58±0·15†	0·19±0·06†	0·38±0·09†	0·22±0·13	75±22
BNF; SO	13·20±7·34‡	1·09±0·54*	2·52±2·28‡	3·83±2·82‡	0·72±0·71	60±16
BNF; H	6·03±2·80†‡	0·28±0·10†*	0·89±0·52†‡	1·65±0·83†‡	0·30±0·34	55±20

B	Total clearance of metronidazole	Clearance to MAA	Clearance to HM	Clearance to GM	Renal clearance	Recovery (% of dose)
Control; SO	5·90±0·88	0·39±0·12	0·19±0·06	0·73±0·35	1·28±0·38	45±14
Control; H	3·64±1·08†	0·20±0·08†	0·07±0·02†	0·23±0·13	1·18±0·28	48±15
BNF; SO	9·16±2·65‡	1·49±0·83‡	0·95±0·50‡	1·52±0·43‡	1·39±0·88	60±14
BNF; H	5·04±1·41†‡	0·64±0·21†‡	0·51±0·19†‡	0·67±0·17†‡	0·89±0·58†	57±17

Control rats and rats pretreated with BNF i.p. (60 mg/kg body weight) received antipyrine i.p. (14 mg/kg body weight) and metronidazole i.p. (11·5 mg/kg body weight). The total clearance and fractional clearances were determined from one saliva sample and urine collected for 20 h, respectively.

All values of clearance are termed as ml/min per kg body weight and are means $\pm$ SD; $n=8$ (control (oil)) and $n=11$ (BNF).

† $p<0.05$ *versus* sham-operation.

‡ The value is larger ($p<0.05$) compared with control.

* The value is minor ($p<0.05$) compared with control.

BNF, β-naphthoflavone in solution with peanut oil; control, peanut oil treatment; SO, sham-operation; H, 70% partial hepatectomy; HMAP, 3-hydroxymethylantipyrine; NORAP, norantipyrine; OHAP, 4-hydroxyantipyrine; MAA, metronidazole acetic acid; HM, hydroxymetronidazole; GM, metronidazole glucuronide; renal, clearance by renal excretion of unchanged compound.

between the post-hepatectomy liver weight and each of the clearance values. None of the regression lines showed a significant intercept with the ordinate axis (table 3 A, figure 1 A). Even though determined with broad confidence intervals all regression lines showed intercept estimates close to zero. In β-naphthoflavone-pretreated rats the slope estimates regarding NORAP and OHAP formation were 4·3 and 5·8 times greater than in non-induced rats, although not statistically significant different (table 3 A).

Metronidazole. The 70% hepatectomy decreased the clearance of metronidazole and the metabolite formation rates to a similar degree, irrespective of β-naphthoflavone pretreatment (table 2 B). There were significant correlations between the residual liver weight and each of the metabolic clearance values. The estimates of intercepts regarding the saliva clearances were approximately 0·4 ml/min. The regression line representing non-induced rats showed a significant intercept with the ordinate axis, but the 95% confidence limits of the intercept included zero for induced rats (table 3 B, figure 1 B). Although determined with broad confidence limits all the regression lines regarding the metabolite formation rates showed intercept estimates close to zero. The slope estimates were not

Table 3. Estimates of linear regression (A) antipyrine and metabolites and (B) metronidazole and metabolites.

	Slope (ml/min per g liver)	Intercept (ml/min)	Correlation coefficient (*r*)
(A) Controls			
Antipyrine	0·112 (0·073–0·151)	−0·075 (−0·380–0·230)	0·86
HMAP	0·039 (0·005–0·073)	−0·019 (−0·289–0·250)	
NORAP	0·012 (−0·001–0·025)	−0·001 (−0·104–0·102)	
OHAP	0·012 (−0·001–0·024)	0·041 (−0·059–0·140)	
Renal	0·004 (−0·003–0·012)	0·028 (−0·034–0·089)	
BNF-treated rats			
Antipyrine	0·224 (0·095–0·354)	−0·142 (−1·478–1·194)	0·63
HMAP	0·021 (0·013–0·030)	−0·063 (−0·154–0·028)	
NORAP	0·051 (0·012–0·091)	−0·135 (−0·544–0·275)	
OHAP	0·069 (0·021–0·119)	−0·091 (−0·599–0·418)	
Renal	0·016 (0·003–0·029)	−0·046 (−0·179–0·087)	
(B) Controls			
Metronidazole	0·080 (0·031–0·129)	0·471 (0·090–0·852)	0·69
MAA	0·007 (0·003–0·012)	0·011 (−0·025–0·047)	
HM	0·005 (0·003–0·007)	−0·007 (−0·020–0·005)	
GM	0·022 (0·012–0·033)	−0·055 (−0·138–1·028)	
Renal	0·010 (−0·004–0·024)	0·199 (0·088–0·310)	
BNF-treated rats			
Metronidazole	0·177 (0·065–0·169)	0·356 (−0·180–0·893)	0·73
MAA	0·024 (0·009–0·038)	−0·005 (−0·158–0·149)	
HM	0·013 (0·004–0·022)	0·030 (−0·064–0·124)	
GM	0·024 (0·015–0·033)	0·001 (−0·092–0·094)	
Renal	0·016 (−0·001–0·032)	0·083 (−0·089–0·255)	

The slope is assigned as clearance per liver weight (ml/min per g) and the intercept as clearance (ml/min). Values in brackets are the 95% confidence intervals.

BNF, β-naphthoflavone in solution with peanut oil; HMAP, 3-hydroxymethylantipyrine; NORAP, norantipyrine; OHAP, 4-hydroxyantipyrine; MAA, metronidazole acetic acid; HM, hydroxymetronidazole; GM, metronidazole glucuronide; renal, clearance by renal excretion of unchanged compound.

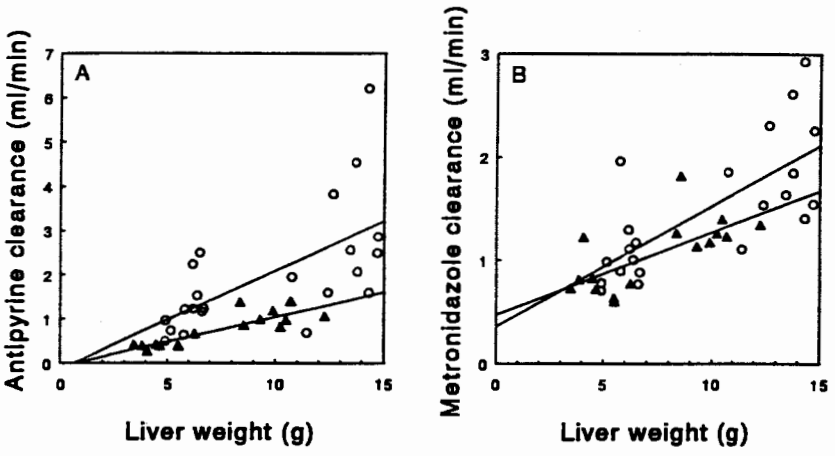

Figure 1. Clearance of (A) antipyrine and (B) metronidazole versus liver weight in rats. Saliva clearances of (A) antipyrine and (B) metronidazole in control rats (▲) and in rats pretreated with β-naphthoflavone (○). The results of the regression analysis are given in table 3.

significantly different between rats with or without β-naphthoflavone pretreatment, but tended to be higher in the latter group with respect to MAA and HM formation (table 3 B).

Discussion

In this study we used partially hepatectomized rats to investigate the possible induction of extrahepatic foreign compound metabolism. The initial hypothesis of the study was that there was no extrahepatic foreign compound metabolism in non-induced rats as indicated by a decrease in liver mass from partial hepatectomy which equalled the decrease in clearance of substances metabolized by the liver (Hansen and Poulsen 1986, Pilsgaard and Poulsen 1984, Poulsen 1985). The alternative hypothesis was that in partially hepatectomized induced rats, a smaller decrease in clearance than that which occurred in liver mass would indicate extrahepatic metabolism.

The principal result of the study was support for the initial hypothesis that, from a quantitative point of view, extrahepatic foreign compound metabolism is negligible, in non-induced as well as in induced rats. From a methodological point of view it was also found that the inductive effect on liver enzymes of β-naphthoflavone is higher after i.p. than after oral administration.

We used antipyrine and metronidazole in a mixture for the determination of foreign compound metabolism. Determination of their clearances from the same saliva sample and metabolite profiles from the urine collected for 20 h is a well studied procedure (Loft *et al.* 1991, 1988). We have shown earlier that in non-induced rats there is a linear relationship between liver weight and antipyrine clearance (Hansen and Poulsen 1986). In the intestine, especially the colon, activity of cytochrome P450 has been demonstrated (Tamura *et al.* 1998), namely, a form inducible by β-naphthoflavone (Oshinsky and Strobel 1987 a, b). In the induced state, especially after oral administration of the inducer compound, a considerable extrahepatic metabolic capacity could possibly emerge.

The present study did not show a significant difference between the intercepts for the two regression lines representing the saliva clearance of control (non-induced)

and induced groups, either when antipyrine or metronidazole are used as probes. Neither were the slopes of the two lines significantly different. Only the intercept for non-induced controls showed a significant intercept with the ordinate axis when metronidazole was used as probe (table 3), but this intercept probably represents the renal clearance, which was unaffected by hepatectomy. No other intercepts obtained in this study could be identified as significantly different from zero. As an attempt to verify the extrahepatic formation of one or more metabolites a linear regression analysis of all the oxidative metabolites was performed, but still none of the intercepts could be identified as significantly different from zero. Glucuronosyltransferase activity towards several substrates is induced in rat liver by PAH-type inducers (Okey 1990) and the glucuronidation of metronidazole represent a substantial part of the elimination pathways. As with the other metabolites, a linear regression analysis did not show a significant intercept, indicating that glucuronidation is exclusively hepatic.

The metabolism of antipyrine and metronidazole and the effect of β-naphthoflavone was similar in intact and sham-operated rats, confirming that neither the anaesthesia nor the surgery interfered with the metabolic pathways of antipyrine or metronidazole. The urinary recovery of antipyrine and metronidazole and their metabolites was similar in sham-operated and partially-hepatectomized groups, indicating that the absorption of these compounds after i.p. administration is not affected by the hepatectomy. The metabolite profiles were similar for both studies compared with previous studies of PAH-type induction in rats (Danhof *et al.* 1979, Loft *et al.* 1991, Rhodes and Houston 1983).

Regeneration of rat liver after partial hepatectomy is a well known phenomenon, and increased amounts of cytochrome P450 follow the regeneration. Previous studies show that the content of cytochrome P450 in the residual liver after partial hepatectomy do not change for the first 36 h after 90% partial hepatectomy (Poulsen 1985). A 20-h urine sample collected immediately after the partial hepatectomy will therefore remain uninfluenced by the regeneration of liver cytochrome P450, and determination of parent compound and metabolites in the urine will represent metabolism after partial hepatectomy.

We conclude, that β-naphthoflavone is more effective as an inducer when given i.p. than when administered orally. Furthermore, from a quantitative point of view β-naphthoflavone does not induce extrahepatic cytochrome P450 activity, i.e. at least not those cytochrome P450s responsible for the metabolism of antipyrine and metronidazole. Thus, the liver is responsible for the major proportion of the elimination of these two compounds in rats.

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