Ethoxyresorufin *O*-deethylation by human liver microsomes

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- 1 As a substrate for human liver microsomes, ethoxyresorufin appears to be metabolised by a group of cytochrome P450 isoenzymes which are inducible by cigarette smoking.
- 2 Kinetic studies in microsomes from four human livers indicate that only one enzyme component is involved over the full substrate range.
- 3 Ethoxyresorufin *O*-deethylation activity at both high and low substrate concentrations correlated with the high affinity component of ethoxycoumarin *O*-deethylation and of diphenyloxazole metabolism.

Keywords ethoxyresorufin human liver microsomes

Introduction

Investigation of the relative activities of individual isoenzymes or families of isoenzymes of cytochrome P450 in human liver requires studies using specific substrate probes which have high affinity for these isoenzymes. Ethoxyresorufin (ethoxyphenoxazone) has been shown to be a specific substrate for the forms of cytochrome P450 inducible by 3-methylcholanthrene in rat liver (Burke & Mayer, 1983). Ethoxyresorufin *O*-deethylation by human liver microsomes as measured at close to saturating substrate concentrations (1 mM) appears to be carried out by a form of cytochrome P450 inducible by smoking (Thompson *et al.*, 1984), and human placental ethoxyresorufin *O*-deethylase is increased 10–30 fold in placentas from smokers (Manchester & Jacoby, 1981).

In this study the apparent Michaelis–Menten kinetics of ethoxyresorufin *O*-deethylation have been determined using microsomes prepared from liver samples from smokers and non-smokers. Also, ethoxyresorufin *O*-deethylation has been measured in parallel with ethoxycoumarin *O*-deethylation and diphenyloxazole metabolism in microsomes from a series of human livers.

Methods

Human liver specimens

Human liver samples surplus to diagnostic requirements and later shown to be histologically normal were obtained at laparotomy from patients undergoing cholecystectomy or vagotomy and pyloroplasty. Ethics committee approval was obtained prior to the study. Patient details are shown in Table 1. Liver was frozen in liquid nitrogen immediately following removal, and stored at –80° C until use.

Preparation of liver microsomes

Liver (200–400 mg) thawed at 4° C was homogenised with a glass/teflon homogeniser (20 s) in 10 ml ice cold buffer (0.25 M potassium phosphate, 0.15 M potassium chloride, 1.0 mM EDTA pH 7.25). Microsomes were then prepared by differential ultracentrifugation as previously described (Woodhouse *et al.*, 1984) except that they were washed once in 0.25 M potassium phosphate buffer pH 7.25 and resedimented before resuspension in 0.25 M potassium phosphate buffer pH 7.5 containing 30% v/v glycerol to a concentration equivalent to approximately

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Table 1 Patient details

<i>Subject</i>	<i>Sex</i>	<i>Age (years)</i>	<i>Drugs</i>	<i>Smoking</i>	<i>Number of cigarettes smoked per day</i>
1	M	60	Nil	N.S.	—
2	F	65	Carbamazepine (200 mg twice daily)	N.S.	—
3	F	65	Pentobarbitone (400 mg)	N.S.	—
4	F	62	Nil	S	10–20
5	M	62	Nil	N.S.	—
6	M	69	Cimetidine (400 mg twice daily)	N.S.	—
7	F	42	Cimetidine	N.S.	—
8	F	59	Nil	N.S.	—
9	M	41	Cimetidine	S	40
10	M	56	Cimetidine (200 mg twice daily)	S	20
11	F	49	Nil	N.S.	—
12	M	66	Nil	N.S.	—
13	F	36	Nil	S	30
14	M	68	Nil	S	?

100 mg liver wet weight ml⁻¹. Microsomes were usually used immediately. However, storage at -80°C until analysis did not alter enzyme activity.

Ethoxyresorufin-O-deethylation (EROD) assay

EROD was measured at 37°C by a modification of the direct fluorimetric assay described by Burke & Mayer (1983). Reactions were carried out in 1 ml fluorimetric cuvettes using a Perkin Elmer model 3000 fluorimeter. Incubations were carried out in a 500 µl final volume containing 300 µg NADPH in 250 µl 10 mM dipotassium hydrogen phosphate buffer (pH 7.5) and up to 250 µl microsomal suspension (containing up to 200 µg protein). Following establishment of a steady baseline (by equilibration for 1 min at 37°C) the reaction was started by addition of 1 µl of ethoxyresorufin in DMSO, the concentration depending on the investigation being carried out. The reaction was quantitated by following the formation of resorufin by monitoring the increase in fluorescence at excitation wavelength 530 nm and emission wavelength 585 nm. The reaction was followed for 2 min over which time it remained linear. Quenching of resorufin fluorescence by microsomal protein was determined for each liver in the kinetic studies and from a quench curve constructed from a larger

liver sample wedge when only small liver samples were available (see below).

EROD kinetics In kinetic studies reactions were carried out at substrate concentrations in the range of 0.03 to 4 µM. At each substrate concentration ethoxyresorufin was added to the incubation mixture in 1 µl dimethylsulphoxide. Duplicate determinations were made at each substrate concentration. Quenching was determined for each liver by measuring the fluorescence of 100 pmol resorufin added in 5 µl dimethylsulphoxide to an incubation mixture containing microsomes in the absence of substrate and cofactors. This gave an accurate estimate of quenching as the resorufin fluorescence was measured against a stable baseline.

Under these incubation conditions the rate of formation of resorufin was linearly related to protein concentrations up to 200 µg per incubation at both high (2 µM) and low (0.03 µM) substrate concentration. Above 200 µg protein per incubation the reaction deviated from linearity probably due to product inhibition by resorufin.

Biopsy sized liver samples (30–50 mg) Microsomes were prepared as described above. EROD activity was measured at substrate concentrations of 2 and 0.03 µM only (the extremes of the

kinetic curve) due to insufficient protein. Incubation conditions were unaltered except that 30–40 μg microsomal protein was used for each incubation and the sensitivity of the fluorimeter was increased. In order to conserve protein from individual microsomal preparations, an overall measure of the protein quenching of resorufin fluorescence was made. Microsomes prepared from a larger wedge were used to construct a quench curve for resorufin (100 pmol) in the presence of 20–240 μg microsomal protein. This was used to determine the appropriate quench factor at the actual protein concentration used. However, quench variation between individuals was negligible (results not shown).

Protein determination Microsomal protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. A micro modification of the assay allowed measurement of 2–10 μg protein.

Ethoxycoumarin-O-deethylation (ECOD) ECOD was measured as described previously (Woodhouse *et al.*, 1983) at high (1000 μM) and low (5 μM) substrate concentrations.

Diphenyloxazole (PPO) metabolism was measured as described previously (Mutch *et al.*, 1985) at high (100 μM) and low (0.1 μM) substrate concentrations.

Results

Kinetics

The kinetics of ethoxyresorufin O-deethylation were studied using hepatic microsomes from four patients (subjects 3, 8, 9 and 10). Kinetic data were analysed firstly by preparing individual Eadie Hofstee plots (V against V/S) and secondly by an iterative nonlinear regression programme (Duggelby, 1981). The Eadie Hofstee plots obtained were all linear (Figure 1 shows data for

Figure 1 A typical Eadie Hofstee plot for ethoxyresorufin O-deethylation by human liver microsomes (Patient 9).

patient 9) and the estimates of $V_{\max}$ and K_m obtained by extrapolation agreed with those obtained from the non linear regression analysis of the data when fitted by a 1 component Michaelis-Menten equation. Computed $V_{\max}$ and K_m values are shown in Table 2 expressed as mean $\pm$ s.d. derived from the mean measured reaction rate at each substrate concentration. It is noticeable that whereas $V_{\max}$ ranged 24 fold between the four individual livers, K_m values varied only two fold.

Microsomes from subjects 9 and 10, both heavy smokers, had higher $V_{\max}$ values than those from subjects 3 and 8, both non-smokers.

Enzyme activities

Table 3 shows enzyme activities in the human liver microsomes studied. EROD was measured at 0.03 μM and 2 μM substrate concentrations in microsomes from 14 small pieces of liver. EROD

Table 2 Ethoxyresorufin-O-deethylation by human liver microsomes: Kinetic parameters derived by iterative regression analysis. Results expressed as mean $\pm$ s.d.

Subject	K_m (μM)	$V_{\max}$ (pmol resorufin formed mg^{-1} microsomal protein min^{-1})
8	0.39 ± 0.08	185 ± 22
3	0.40 ± 0.07	60 ± 5.4
9	0.24 ± 0.05	860 ± 90
10	0.48 ± 0.03	1442 ± 70

Table 3 Microsomal monooxygenases in human liver microsomes

Subject	Ethoxyresorufin-O-deethylation ($\text{pmol mg}^{-1} \text{microsomal protein min}^{-1}$) $0.03 \mu\text{M}^*$	Ethoxycoumarin-O-deethylation ($\text{pmol mg}^{-1} \text{microsomal protein min}^{-1}$) $5 \mu\text{M}^*$	Ethoxycoumarin-O-deethylation ($\times 10^3 \text{ mg}^{-1} \text{microsomal protein min}^{-1}$) $0.1 \mu\text{M}^*$	PPO metabolism fluorescence units $100 \mu\text{M}^*$
1	14.0	155.5	3.7	159.5
2	29.9	341.9	18.8	219.5
3	9.5	39.5	11.8	251.8
4	11.4	320.5	10.7	215.2
5	41.4	532.2	11.0	120.7
6	0	14.5	2.6	87.4
7	0	36.4	4.1	115.3
8	13.0	280.0	—	94.0
9	61.0	567.1	19.2	170.0
10	119.5	1430.3	—	—
11	7.0	135.1	5.2	165.5
12	75.6	382.1	14.0	138.7
13	29.0	215.6	7.9	117.3
14	89.9	479.2	17.3	207.4

*Substrate concentration.

ranged from 0 to 120 at $0.03 \mu\text{M}$ and from 14.0 to 1450 (pmol resorufin formed mg^{-1} microsomal protein min^{-1}) at $2 \mu\text{M}$. EROD activity was greater in liver microsomes from the five smokers (602.5 ± 215.7 , mean $\pm$ s.e. mean) compared to the 10 non smokers (223.8 ± 70.8) ($P < 0.05$ Mann Whitney U test). In smokers there was no relation between EROD activity and their declared smoking habits.

ECOD was measured also at low and high substrate concentrations in twelve of the livers. The range of activity was less than for EROD, ranging from 3 to 20 pmol mg^{-1} microsomal protein min^{-1} at $5 \mu\text{M}$ and from 87 to 250 pmol mg^{-1} microsomal protein min^{-1} at $1000 \mu\text{M}$. PPO metabolism was also measured in six livers and ranged from 3.5 to 45.2 fluorescence units $\times 10^3 \text{ mg}^{-1} \text{ min}^{-1}$ at $0.1 \mu\text{M}$ and from 154 to 499 FU $\times 10^3 \text{ mg}^{-1} \text{ min}^{-1}$ at $100 \mu\text{M}$.

The correlations (r) between EROD, ECOD and PPO metabolism are shown in Table 4. EROD activity at $0.03 \mu\text{M}$ correlated well with that at $2 \mu\text{M}$ ($P < 0.01$) as shown in Figure 2. ECOD activity measured at low substrate ($5 \mu\text{M}$) correlated with EROD at both low substrate concentration ($P < 0.01$) and high substrate ($P < 0.01$). However, ECOD activity measured at high substrate did not correlate with EROD activity at high or low substrate or with ECOD activity at low substrate. Similarly PPO metabolism at low substrate concentration correlated with EROD at both high ($P < 0.05$) and low substrate ($P < 0.01$). Metabolism at high substrate did not parallel EROD activity but correlated with both high substrate ($P < 0.01$) and low substrate ($P < 0.05$) ECOD.

Figure 2 The relationship between ethoxyresorufin *O*-deethylation activities at low ($0.03 \mu\text{M}$) substrate and high ($2 \mu\text{M}$) substrate concentration.

Discussion

Ethoxyresorufin is metabolised by human liver microsomes at widely varying rates with a 100 fold range in activity when measured at $2 \mu\text{M}$ substrate. EROD activity was greater in microsomes from smokers than from non-smokers. EROD kinetics were investigated with microsomes from four human livers; the highest V_{max} values were in livers 9 and 10 from smokers whereas livers 3 and 8 were from non-smokers. Furthermore the lowest V_{max} was measured with microsomes from a patient receiving pentobarbitone (Liver 3). Therefore, as previously suggested (Thompson *et al.*, 1984), ethoxyresorufin *O*-deethylation appears to be carried

Table 4 Correlations between ethoxyresorufin-*O*-deethylation, ethoxycoumarin-*O*-deethylation and PPO metabolism

Enzyme (substrate concentration)	EROD 0.03 μM	EROD 2 μM	ECOD 5 μM	ECOD 1000 μM	PPO 0.1 μM	PPO 100 μM
EROD 0.03 μM		0.87** ($n = 14$)	0.74** ($n = 12$)	0.18 ($n = 13$)	0.96** ($n = 6$)	0.27 ($n = 6$)
	EROD 2 μM		0.77** ($n = 12$)	0.14 ($n = 13$)	0.83** ($n = 6$)	0.16 ($n = 6$)
		ECOD 5 μM		0.57 ($n = 12$)	0.59 ($n = 6$)	0.83* ($n = 6$)
			ECOD 1000 μM		-0.06 ($n = 6$)	0.97** ($n = 6$)
				PPO 0.1 μM		0.10 ($n = 6$)
					PPO 100 μM	

** $P < 0.01$, * $P < 0.05$.

out by a cytochrome P450 isoenzyme or group of isoenzymes which are induced by cigarette smoke and not by pentobarbitone.

Despite the wide differences in $V_{\max}$ and activity, K_m values varied only two fold between individuals and appeared to be similar in smokers and non-smokers. These findings suggest that the isoenzymes involved in EROD in smokers' liver have similar affinities for ethoxyresorufin to those in non-smokers' liver and may be different amounts of the same isozymes. EROD has been shown to be a highly specific reaction when mediated by rat microsomes, being induced 50–70 fold by 3-methylcholanthrene although the K_m was unaltered (Burke & Mayer, 1984). Cigarette smoking induces the same isoenzymes (Thompson *et al.*, 1984).

The linear Eadie Hofstee plots suggest that one isoenzyme or group of isoenzymes of similar substrate affinities mediate the reaction over a 100 fold range of substrate concentrations. The high correlation between activity at low substrate and high substrate in the liver samples studied support this conclusion. These findings contrast with our earlier observations of ethoxycoumarin *O*-deethylation (Woodhouse *et al.*, 1984) and PPO metabolism (Mutch *et al.*, 1985) by human liver microsomes when a non linear Eadie Hofstee plot indicated the involvement of more than one group of isoenzymes, which could be distinguished at high and low substrate concentrations. In this study ethoxyresorufin *O*-deethylation

correlated with the high affinity component of ECOD and PPO metabolism. The correlation between activities may imply metabolism by a common group of cytochrome P450 isoenzymes. If this is the case one would expect the high affinity components of ECOD and PPO metabolism to be induced by cigarette smoking to the same extent as EROD. However our correlations were based on activities in microsomes from nonsmokers and the only smoker included did not appear to have induced activity. Studies in more smokers should elucidate the problem. The correlation coefficients obtained indicate that not all of the variance in ECOD or PPO activity can be accounted for by variance in EROD activity. Birgesson *et al.* (1985) have also found a weak correlation between debrisoquine hydroxylation and EROD activity in human liver microsomes. The low affinity components of ECOD and PPO metabolism did not correlate with EROD, suggesting the involvement of different cytochrome P450 isoenzymes. In support of this we have found no correlation between smoking habit and the low affinity content of ECOD.

In conclusion, ethoxyresorufin appears to be metabolised by a single isoenzyme, or by a group of isoenzymes with similar affinities and to be inducible by smoking.

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