Debrisoquine-type polymorphism of drug oxidation: purification from human liver of a cytochrome P450 isozyme with high activity for bufuralol hydroxylation

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Indirect evidence suggests that the genetically defective metabolism of drugs such as debrisoquine and bufuralol observed in up to 10% of the population (poor metabolizers) is caused by the absence or functional deficiency of a cytochrome P450 isozyme. Using bufuralol-1'-hydroxylation to carbinol to optimize the procedure, 3 cytochrome P450 isozymes (P450A, P450buf, P450C) were purified to apparent electrophoretic homogeneity from human liver microsomes. P450buf had a specific activity of 20.3 nmol carbinol·nmol $P450^{-1}\cdot 15\ min^{-1}$ as compared to microsomes (10.0 nmol carbinol·nmol $P450^{-1}\cdot 15\ min^{-1}$) when (+)-bufuralol was used as substrate. The stereoselective metabolism of (−)- and (+)-bufuralol to carbinol by purified P450buf [(−)/(+) ratio: 0.13] was strikingly different from that in the microsomes of either an extensive [(−)/(+) ratio: 0.4] or poor metabolizer [(−)/(+) ratio: 0.83] of bufuralol. We propose that this isozyme is the major bufuralol and debrisoquine hydroxylating species and is the target of the genetic deficiency.

Cytochrome P450 *Functional deficiency* *Debrisoquine polymorphism* *Human liver* *Bufuralol-1'-hydroxylation* *Stereoselectivity*

1. INTRODUCTION

Drug biotransformation by hepatic polysubstrate monooxygenases (trivial name: cytochrome P450) is a major determinant of the therapeutic and toxic responses to a broad variety of clinically important drugs. Recent indirect evidence from this and other laboratories suggest that a common genetic polymorphism causing impaired biotransformation of debrisoquine, sparteine, bufuralol and a number of other drugs [1–7] is caused by the absence or functional deficiency of a cytochrome P450 isozyme [8–10]. This polymorphism occurs with a frequency of up to 10% in European populations and separates individuals into 'extensive (EM' and 'poor (PM)' metabolizer phenotypes for the metabolism of these drugs. The defect is inherited as an autosomal-recessive trait [11]. Its clinical relevance has been demonstrated [4,5,12].

To elucidate the molecular basis of this genetic defect, the cytochrome P450 isozyme(s) responsible for this monooxygenase activity has (have) to be isolated from human tissue and characterized.

2. EXPERIMENTAL

Microsomes from 300 g (wet wt) liver of kidney transplant donors (KDL) were prepared as in [8]. The purification of a cytochrome P450 with high bufuralol hydroxylation activity (P450buf) was achieved by following the reconstituted specific activity of bufuralol-1'-hydroxylation to carbinol of the individual fractions. Briefly, after cholate solubilization a microsomal supernatant containing approx. 1000 nmol P450 was applied to an ω-aminooctyl Sepharose-4B column (3.6 × 30 cm, pH 7.25 [13]). P450-containing fractions were

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eluted with buffer containing 0.06 and 0.5% (w/v) Emulgen 913, respectively [13,14]. The latter fraction was then applied to a hydroxyapatite column (2.6 × 8 cm, pH 7.25) and P450 fractions were eluted stepwise with 40, 90, 180 and 300 mM potassium phosphate [14]. After dialysis the fraction obtained at 90 mM phosphate was loaded onto a DE-Sepharose CL-6B-column (1.6 × 10 cm, pH 7.8) and a linear gradient of 0–250 mM NaCl led to the elution of P450buf at ~40 mM NaCl. Cytochrome P450buf showed a single homogeneous band on SDS–PAGE when 5 μg protein were applied to a 1 mm gel. Furthermore, two other electrophoretically homogeneous cytochromes, P450A and P450C, were purified from one liver. They were obtained by chromatography on DE-Sepharose CL-6B of the fractions eluting at 90 and 180 mM phosphate from the hydroxyapatite column.

NADPH cytochrome P450 reductase (P450 reductase) was purified from rat or human liver microsomes as in [15]. The preparations had specific activities of 40 units/mg (nmol cytochrome *c* reduced · min^{-1}, RT) and 10 units/mg, respectively. They could be used interchangeably in reconstitution experiments.

Detergents were removed from the purified protein fractions with Bio-Beads SM-2 [16]. For reconstitution of monooxygenase activity 10–20 pmol P450, 0.3 units P450 reductase, 20 μg/ml of dilauroyl-L-α-phosphatidylcholine, 5 mM $MgCl_2$, 5 mM isocitrate, 1 unit isocitrate dehydrogenase, 1 mM NADPH and the indicated substrate concentration were incubated in a final volume of 250 μl of 0.1 M potassium phosphate (pH 7.4), 20% (v/v) glycerol. The system was preincubated at 4°C for 30 min prior to addition of the NADPH-regenerating system and at 37°C for 5 min before addition of the substrate. After 15 min of incubation at 37°C with gentle shaking, a 100 μl aliquot was mixed with 10 μl of 12% (w/v) $HClO_4$, proteins were sedimented and the supernatant stored at −80°C until analysis. The carbinol metabolite was determined by reversed phase HPLC on a Spherisorb ODS (5 μm) column with fluorescence detection at the excitation/emission wavelength pair of 252/303 nm. The rate of bufuralol-1′-hydroxylation was linear with time up to 20 min and with P450buf up to 25 pmol/250 μl. The reaction was dependent on NADPH and inhibited by CO. NADH did not support the reaction. When the bufuralol-1′-hydroxylation assay was carried out in microsomes, 200 μg microsomal protein in 1 ml of 0.1 M sodium phosphate (pH 7.4) was incubated.

For screening activities in individual fractions at early stages of the purification (table 1) the assay was not optimized for linearity with time and protein concentration. This explains the different activities for carbinol production by P450buf in table 1 *vs* table 2.

Ethoxycoumarin-*O*-deethylase activity was measured as in [17] with the reconstituted system containing 50 pmol P450 isozymes and P450 reductase (0.8 units) in 1 ml final volume. Benzphetamine-*N*-demethylation was determined using 150 pmol P450 and 0.4 units P450 reductase in 300 μl final volume [18]. Both activities were linear with time and protein content. P450 concentration was determined by CO-binding spectra [19]. Protein was determined according to [20]. Racemic, (+)-, (−)-, and 1′-OH-bufuralol carbinol were kindly provided by Hoffmann-La Roche, Basel, Switzerland, and Welwyn Garden City, England (Dr R. Francis).

3. RESULTS AND DISCUSSION

By monitoring the activity of bufuralol-1′-hydroxylation in the individual column eluates we have developed a procedure which results in the reproducible purification from different human livers of a P450 isozyme (P450buf) with a high turnover number for this reaction. P450buf was homogeneous on SDS–PAGE but apparently had lost some prosthetic heme resulting in a decreased heme–CO binding complex in relation to the protein content (table 1). Two additional isozymes with low turnover numbers for bufuralol-1′-hydroxylation and variant activities towards benzphetamine and ethoxycoumarin also were purified from one liver (table 2).

Many monooxygenase reactions are characterized by stereospecific recognition of substrates with asymmetric carbon centers. When the pure enantiomers of bufuralol were used as substrates, the reconstituted P450buf showed strikingly increased selectivity for the (+) enantiomer with a 3–4-fold lower (−)/(+) ratio than the microsomes from the same extensive metabolizer liver (table 3).

Table 1

Purification of cytochrome P450buf from human liver

	Protein (mg)	P450 (nmol)	Specific content (nmol P450/ mg protein)	Yield (% P450)	nmol carbinol· $nmol^{-1}$ P450·h^{-1}
Microsomes	1025	947	0.92	100	6.0
ω-Aminooctyl Sepharose-4B (eluate at 0.5% Emulgen)	89	163	1.83	17.2	1.4
Hydroxyapatite (eluate at 90 mM phosphate)	15.5	20.2	1.3	2.1	5.1
DE-Sepharose CL-6B (eluate at 40 mM NaCl)	8.4	9.6	1.13	1.0	16.8

Bufuralol-1'-hydroxylation was carried out in a system containing 150 pmol P450, 0.8 units NADPH cytochrome P450 reductase and 1 mM racemic bufuralol as substrate. Incubation was for 1 h. The other assay conditions were as described in the text

Thus, in P450buf a highly stereoselective component of the reaction was isolated. By contrast, microsomes of a poor metabolizer demonstrated minimal stereoselectivity consistent with the absence or functional deficiency of this stereoselective component.

Moreover, whereas the rate of formation of carbinol from the (−)-bufuralol was similar for P450buf, for extensive metabolizer and poor metabolizer microsomes the carbinol production rate from (+)-bufuralol showed marked differences (table 3).

These findings suggest a functional deficiency or a lack of P450buf specific for (+)-bufuralol-1'-hydroxylation in poor metabolizer livers as the cause of the debrisoquine-type polymorphism. The purified isozyme will now serve to develop immunological and gene probes to investigate the molecular basis of this common defect of drug metabolism.

Table 2

Molecular mass, specific content and enzymatic activities of 3 purified cytochrome P450 isozymes

	Molecular mass (kDa) (SDS–PAGE)	Specific content (nmol P450· mg^{-1} protein)	Substrates		
			Bufuralol[a] (nmol carbinol· $nmol^{-1}$ P450· 15 min^{-1})	d-Benzphetamine (nmol HCHO· $nmol^{-1}$ P450·min^{-1})	7-Ethoxycoumarin (pmol 7-OH-coumarin· $nmol^{-1}$ P450·min^{-1})
P450buf[b]	50	1.13	8.7	4.1	n.d.
P450A[c]	55	4.6	n.d.	7.2	41.0
P450C	55	2.25	0.49	5.1	n.d.

[a] Racemic mixture of bufuralol
[b] P450buf was purified from kidney donor livers 6 and 8
[c] P450A and P450C were purified from kidney donor liver 6
[d] n.d., not detectable

Table 3

Stereoselective metabolism of (+)- and (−)-bufuralol by extensive (EM)-, poor (PM)-metabolizer microsomes and reconstituted P450buf

	(−)-Bufuralol (640 μM) (nmol carbinol · nmol^{-1} P450 · 15 min^{-1})	(+)-Bufuralol (640 μM) (nmol carbinol · nmol^{-1} P450 · 15 min^{-1})	(−)/(+) ratio
Reconstituted P450buf	2.65 (2.5 −2.8)[a]	20.3 (16.1–24.5)[a]	0.13 (0.11–0.15)
EM-microsomes	3.75 (4.04–3.46)[a]	10.0 (7.2–12.9)[a]	0.405 (0.5 −0.31)
PM-microsomes	2.0[b]	2.4	0.83

[a] Values in parentheses denote the range of V_{max} derived from two experiments in which the concentration of substrate was varied from 1 to 640 μM

[b] Only a single experiment was done because of a limited amount of tissue

Assay conditions are as described except that the incubation time for microsomes was 40 min.

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