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CHARACTERIZATION OF A COMMON GENETIC DEFECT OF CYTOCHROME P-450 FUNCTION (DEBRISOQUINE-SPARTEINE TYPE POLYMORPHISM) - Increased Michaelis Constant (Km) and Loss of Stereoselectivity of Bufuralol 1'-Hydroxylation in Poor Metabolizers

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SUMMARY: In order to define the mechanism of the debrisoquine-sparteine type genetic polymorphism of drug oxidation we studied the kinetics of bufuralol 1'-hydroxylation in liver microsomes from extensive and poor metabolizers and in a purified reconstituted human cytochrome P-450 isozyme with high activity for bufuralol 1'-hydroxylation, P-450[buf]. In extensive metabolizer microsomes the enzymatic reaction displayed apparent Michaelis-Menten kinetics and the (+)-isomer was preferentially metabolized. By contrast, the enzymatic reaction in poor metabolizer microsomes was characterized by a 4- to 5-fold increase in Km and by a loss of stereoselectivity. In a non-membraneous reconstituted system containing NADPH cytochrome P-450 reductase, a NADPH regenerating system and phospholipids, P-450[buf] exhibited an almost complete substrate stereoselectivity for (+)-isomer 1'- hydroxylation. It is concluded that the purified cytochrome P-450[buf] is the target of the debrisoquine-sparteine type oxidation polymorphism and that poor metabolizers have a quantitative or qualitative deficiency of this isozyme.

Biotransformation by hepatic polysubstrate monooxygenases (cytochrome P-450) is a major determinant of the interindividual differences in the rate of elimination of numerous xenobiotics and endogenous substances. Among the causes of this variability the recently discovered genetic polymorphism of debrisoquine and sparteine metabolism (1-4) is of considerable clinical relevance (4-6). Poor metabolizer subjects who represent 3-10% of the population in Europe and North America (2,4,7) have an impaired oxidation of numerous other drugs including bufuralol (8). Family studies indicate that poor metabolizers are homozygous for an autosomal recessive gene (1,2,4,8). Biochemical investigations have provided indirect evidence that the enzymatic reactions which are deficient in poor metabolizers are mediated by cytochrome P-450 hemoprotein monooxygenases (9-11). We have demonstrated that the in vivo oxidative metabolism of bufuralol is under the

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same genetic control as the metabolism of debrisoquine (8). More recently, this laboratory has shown that the human microsomal oxidation of bufuralol correlates also with that of debrisoquine (11).

In order to define the mechanism of the debrisoquine-sparteine type polymorphism we have now investigated the enzyme kinetics of the 1'-hydroxylation of bufuralol in liver microsomes from subjects previously phenotyped as extensive and poor metabolizers for debrisoquine or sparteine. The kinetics of bufuralol 1'-hydroxylation were also monitored in a non-membraneous reconstituted system containing an isozyme from human liver with high activity for bufuralol 1'-hydroxylation purified in this laboratory (12).

MATERIALS AND METHODS

Microsomes: Human liver samples were obtained as wedge biopsies at laparotomy for diagnostic or therapeutic reasons or from kidney transplant donors (10). Patients undergoing wedge biopsy were phenotyped and characterized in vivo as extensive (EM, n=4) and poor (PM, n=2) metabolizers using debrisoquine (2) or sparteine (3). Wedge biopsies were immediately frozen in liquid nitrogen and kept at -80°C. For preparation of microsomes small samples (100-150 mg wet weight) were homogenized in 0.5 ml of 0.25 mol/L sucrose, pH 7.4, 3 times for 30 sec at 17.000 rpm (Polytron homogenizer, Kinematica, Kriens, Switzerland) interrupted by 1 min cooling intervals (11). The whole procedure was carried out in the cold room (< 5°C). The homogenate was centrifuged in an Eppendorf 5414 centrifuge at 10.000 x g for 5 min. The pellet was resuspended in 0.5 ml sucrose and again centrifuged. Both supernatants were combined and centrifuged for 12 min in a Beckman Airfuge at 148.000 x g . The pellets were resuspended in 0.1 mol/L Na-pyrophosphate, pH 7.4, again centrifuged for 12 min at 148.000 x g and the final pellets resuspended in 0.1 mol/L $NaPO_4$, pH 7.4, and stored at -80°C.

The protein content of the various microsomal preparations ranged from 3.1 to 4.7 mg/ml (13) and the content of spectrally measured cytochrome P-450 from 0.28 to 0.44 nmol cytochrome P-450/mg protein (14). Microsomes from larger liver pieces from kidney donors were prepared as described (12). Microsomes were washed twice in Na-pyrophosphate 0.1 mol/L, resuspended in 0.1 mol/L $NaPO_4$, pH 7.4 and then kept at -80°C until assayed.

Cytochrome P-450 purification: The purification was achieved by following bufuralol 1'-hydroxylation activity in eluates during chromatography as described (12). After cholate solubilization the microsomal supernantant was applied to an Amino-octyl Sepharose-4B column. Various P-450 fractions were eluted with buffers containing 0.06 % (w/v) and 0.5 % (w/v) Emulgen 913. Fractions with higher activity were applied to a hydroxylapatite column and cytochrome P-450 fractions eluted stepwise with 40, 90, 180 and 300 mmol/L KPO_4 (15). After dialysis the fraction obtained at 90 mmol/L KPO_4 had the highest activity and was applied on a DE-Sepharose CL-6B column. Subfractions were eluted with a linear gradient of 0 to 250 mmol/L NaCl. An isozyme with a high activity for bufuralol 1'-hydroxylation was eluted at approximately 40 mmol/L NaCl and displayed a single homogeneous band on SDS-PAGE (12).

Assay for bufuralol 1'-hydroxylation: Fifty ug of microsomal protein (10-20 pmol cytochrome P-450) were incubated in a final volume of 250 ul of 0.1 mol/L

$NaPO_4$, pH 7.4, containing a NADPH regenerating system (NADPH 1 mmol/L, $MgCl_2$ 5 mmol/L, isocitrate 5 mmol/L, isocitrate dehydrogenase 1 Unit). The system was preincubated at 37°C for 5 min before addition of (+)- or (-)-bufuralol (final concentrations 5 to 640 umol/L) and incubated for 40 min at 37°C. Under these conditions the rate of 1'-hydroxy-bufuralol formation was linear with time for up to 1 hr. The reaction was stopped by cooling and addition of 20 ul of 60% $HClO_4$. Proteins were sedimented by centrifugation and supernatants stored at -80°C until analysis.

For reconstitution of monooxygenase activity with purified proteins, detergents were removed with Bio-Beads SM-2 (16). Then 10-20 pmol cytochrome P-450, 0.3 Units of NADPH cytochrome P-450 reductase, 20 ug/ml L-alpha-phosphatidylcholine dilauroyl and a NADPH regenerating system (see above) were incubated for 15 min in a final volume of 250 ul of 0.1 mol/L KPO_4, pH 7.4, containing 20% (v/v) glycerol. Final concentrations of (+)- or (-)-bufuralol ranged from 1 to 320 umol/L. NADPH cytochrome P-450 reductase purified from rat or human liver microsomes (17) could be used interchangeably. The reaction was stopped as described for microsomes.

1'-hydroxybufuralol production was determined in $HClO_4$-supernatants by reverse-phase liquid chromatography. Aliquots (20 ul) of the $HClO_4$-supernatants were injected on a Spherisorb ODS (5 um) column, the mobile phase was 20 mmol/L $NaClO_4$-acetonitrile (55/45, v/v). Column effluent was monitored with a fluorescence detector (Perkin Elmer 650-10S) at an excitation/emission wavelength pair of 252/303 nm and quantitated by external standardization.

Statistics: The untransformed data were analyzed by means of a non-linear least square curve fitting program which allows an observer-independent weighting of the data (18). Simple Michaelis-Menten type kinetics offered the best description of the results in the substrate range covered throughout the study. At higher concentrations (> 10^{-3} mol/L of (+)-bufuralol) inhibition was observed. The non-parametric Wilcoxon-Mann-Whitney test was applied for group comparison .

RESULTS AND DISCUSSION

The stereoselectivity of the enzymatic reactions of microsomal polysubstrate monooxygenases frequently allows a more accurate definition of the isozyme (s) involved. Bufuralol, with its chiral carbon center and fluorescent metabolites offers a sensitive tool to investigate the metabolic pathway(s) under genetic control known as debrisoquine-sparteine polymorphism. Moreover, bufuralol metabolism displays an apparent simple Michaelis-Menten type of 1'-hydroxylation kinetics (11) which is not the case with other prototype compounds such as debrisoquine (19). The substrate stereospecificity of bufuralol 1'-hydroxylation was studied in microsomes prepared from extensive and poor metabolizers as well as in the reconstituted purified isozyme (Figure 1). The relative amounts of 1'-hydroxybufuralol formed from (-)- and (+)-bufuralol ((-)/(+) ratios) in microsomes of extensive metabolizers ranged from 0.32 to 0.63 (Table I), demonstrating a preferential 1'-hydroxylation of the (+)-isomer. By contrast poor metabolizer microsomes were characterized by a striking loss of this stereoselectivity with

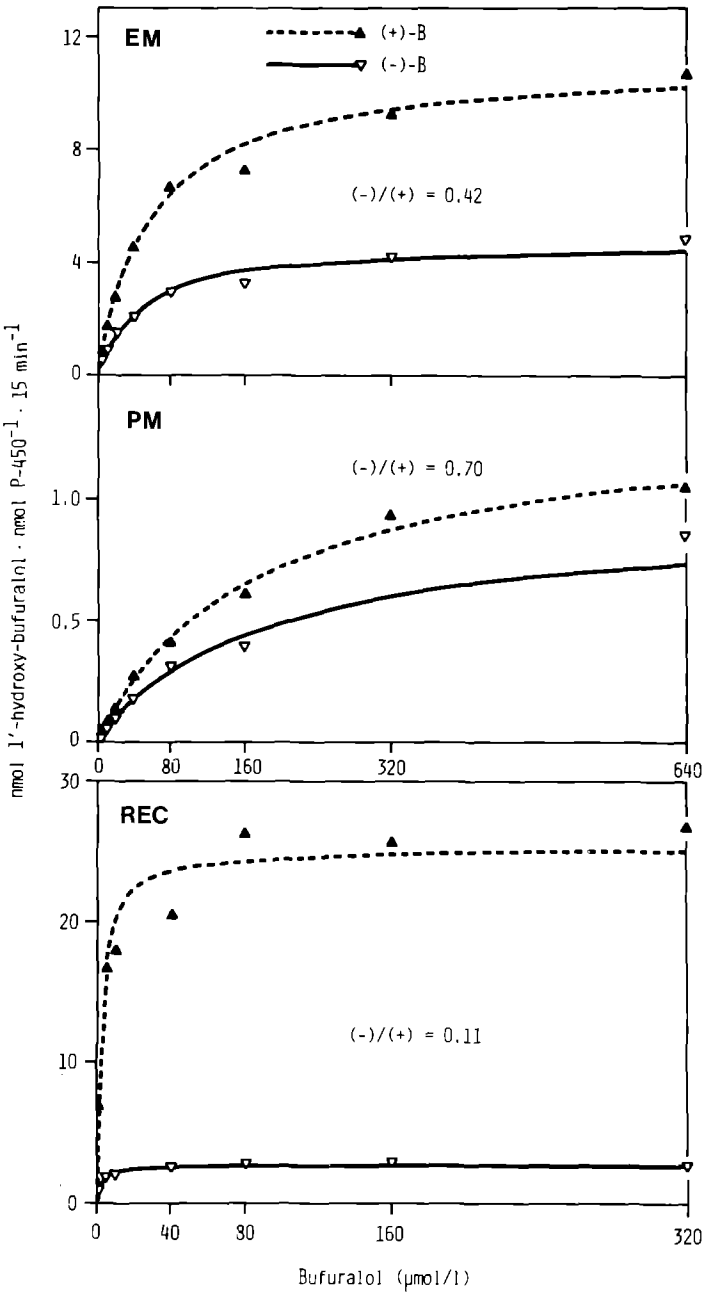

Figure 1: (+)- and (-)-bufuralol 1'-hydroxylation in human liver microsomes from an extensive (EM) and a poor (PM) metabolizer and in a non-membraneous reconstituted system (REC) containing a purified cytochrome P-450 from human liver with a high activity for bufuralol 1'-hydroxylation.

(-)/(+) ratios of 0.70 and 0.83. These observations are in agreement with the known *in vivo* stereoselectivity of bufuralol 1'-hydroxylation in extensive metabolizers (20) and the disappearance of this component in poor metabolizers

TABLE I: Kinetic parameters of (+)- and (-)-bufuralol 1'-hydroxylation in microsomes of extensive (EM) and poor (PM) metabolizers

Phenotype	(+)-bufuralol		(-)-bufuralol		(-)/(+) ratio[+]	in vivo metabolic ratio (sparteine)°
	Km*	Vmax¶	Km*	Vmax¶		
EM_1	40.6	8.6	21.8	2.7	0.31	0.2
EM_2	40.6	5.1	32.4	2.9	0.57	0.4
EM_3	57.2	11.2	44.7	4.7	0.42	0.5
EM_4	50.6	9.6	42.2	6.0	0.63	0.8
$\bar{m}$	47.3	8.6	35.3	4.1	0.48	-
(SD)	(8.1)	(2.6)	(10.4)	(1.6)	(0.15)	-
PM_1	193	2.4	287	1.99	0.83	66
PM_2	172	1.3	180	0.93	0.70	43

* umol/L, ¶ nmol 1'-hydroxybufuralol x nmol P-450^{-1} x 15 min^{-1}; + ratio of (-) Vmax/(+) Vmax; ° *in vivo* sparteine urinary metabolic ratio (antimode at 20) (3) with the exception of PM_1 who was phenotyped with the debrisoquine urinary metabolic ratio (antimode at 12.6) (2).

(21). The fact that Vmax values for (-)-bufuralol 1'-hydroxylation in extensive metabolizers are similar to Vmax values for (+)- and (-)-bufuralol hydroxylation in poor metabolizers (Table I) suggests that (-)-bufuralol 1'-hydroxylation might be, at least in part, catalyzed by other enzyme(s) not related to the genetic polymorphism. This hypothesis is further supported by the increase in substrate selectivity observed in the non-membraneous reconstituted system containing the purified isozyme (Table II). The increased substrate stereoselectivity and the low Km values support the assumption that the purified isozyme P-450[buf] represents the target of the genetic variation.

The kinetic parameters of the enzymatic reactions show little variability within each phenotype. Using (+)-bufuralol as substrate there is no overlap of the apparent Vmax (Figure 2) or the Km (Table I) between the poor and extensive metabolizers tested. These two parameters, when combined with the enantiomer ratio, appear to offer an interesting approach which allows to phenotype individuals *in vitro* until appropriate specific antibodies become available.

TABLE II: Kinetic parameters of (+)- and (-)-bufuralol 1'-hydroxylation in human liver microsomes and in a non-membraneous reconstituted system containing a purified cytochrome P-450 isozyme from the same kidney donor liver

	(+)-bufuralol		(-)-bufuralol		
	Km*	Vmax¶	Km*	Vmax¶	(-)/(+) ratio+
Microsomes	28.3	7.7	30.4	3.5	0.45
P-450[buf]° a)	2.9	16.1	2.0	2.5	0.15
b)	2.6	25.0	2.0	2.8	0.11

* umol/L, ¶ nmol 1'-hydroxybufuralol x nmol P-450^{-1} x 15 min^{-1}; + ratio of (-) Vmax/(+)Vmax; ° two reconstitution experiments with the same purified fraction. Microsomes prepared as in (12): the Km values are therefore not comparable to those in Table I.

We conclude that a quantitative or qualitative deficiency of this highly stereoselective cytochrome P-450[buf] is likely to be the cause of this common genetic variation of drug oxidation. An isozyme-specific antibody will be required to test for the presence or absence of the corresponding isozyme in poor metabolizer liver. Whether the high Km for bufuralol in microsomes of poor metabolizers is due to a structural variant of cytochrome P-450[buf] or reflects metabolism by other less specific microsomal isozyme(s) not affected by the polymorphism is presently under investigation.

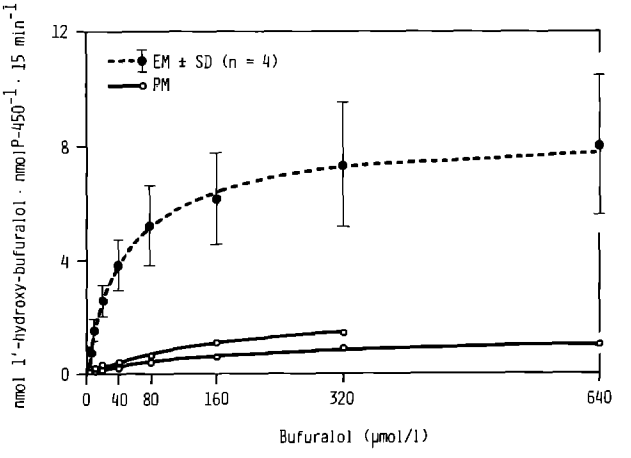

Figure 2: (+)-bufuralol 1'-hydroxylation kinetics in human liver microsomes from extensive (EM) and poor (PM) metabolizers phenotyped in vivo.

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