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Absence of Hepatic Cytochrome P450bufI Causes Genetically Deficient Debrisoquine Oxidation in Man†

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Received December 7, 1987; Revised Manuscript Received March 16, 1988

ABSTRACT: The common genetic deficiency of drug oxidation known as debrisoquine/sparteine-type polymorphism was investigated with bufuralol as prototype substrate. In human liver microsomes the 1′-hydroxylation of bufuralol is catalyzed by two functionally distinct P-450 isozymes, the high-affinity/highly stereoselective P450bufI and the low-affinity/nonstereoselective P450bufII. We demonstrate that P450bufI is unique in hydroxylating bufuralol in a cumene hydroperoxide (CuOOH) mediated reaction whereas P450bufII is active only in the classical NADPH- and O_2-supported monooxygenation. In microsomes of liver biopsies of in vivo phenotyped poor metabolizers of debrisoquine or sparteine, the CuOOH-mediated activity was drastically reduced. Rabbit antibodies against a rat P-450 isozyme with high bufuralol 1′-hydroxylase activity (P450db1) precipitated exclusively P450bufI-type activity from solubilized microsomes. Western blotting of microsomes with these antibodies revealed a close correlation between the immunoreactive protein and CuOOH-mediated (+)-bufuralol 1′-hydroxylation. No immunoreactive protein was detected in liver microsomes of in vivo phenotyped poor metabolizers. These data provide evidence for a specific deficiency of P450bufI and are consistent with the complete or almost complete absence of this protein in the liver of poor metabolizers.

Cytochrome P-450 (P-450)[1] is the collective term for a group of hemoprotein isozymes with broad and overlapping substrate specificities responsible for the oxidative metabolism of a large number of endobiotic and xenobiotic substances (Ortiz de Montellano, 1986; Meyer, 1984; Adesnik & Atchison, 1986). In recent years, several genetically determined polymorphisms of P-450-mediated drug oxidation have been discovered (Idle & Smith, 1979; Küpfer & Preisig, 1983; Eichelbaum, 1984). These polymorphisms cause impaired biotransformation of certain drugs in so-called "poor metabolizer" or PM subjects. Because of their frequency of occurrence, genetic polymorphisms of drug oxidation are a major determinant of interindividual differences in the therapeutic and toxic responses to numerous clinically important drugs.

One of the most extensively studied examples of a genetically determined variation in drug metabolism is the debrisoquine/sparteine-type polymorphism which occurs in up to 10% of individuals in Caucasian populations and affects the metabolism of over 20 clinically used drugs including debrisoquine, sparteine, and bufuralol (Price-Evans, 1986; Meyer et al., 1986). Pedigree studies suggest that this defect is monogenically inherited as an autosomal recessive trait (Eichelbaum et al., 1979; Price-Evans et al., 1980). Numerous studies have been undertaken to evaluate the molecular basis of this important polymorphism, but so far without success.

Investigations of the metabolism of debrisoquine or bufuralol in microsomes of liver biopsies of in vivo phenotyped extensive and poor metabolizer subjects have supported the hypothesis

† Supported by Grant 3.817.87 of the Swiss National Science Foundation.
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[1] Abbreviations: P-450, cytochrome P-450; CuOOH, cumene hydroperoxide; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate; EM, extensive metabolizer (phenotype); PM, poor metabolizer (phenotype); KDL, kidney donor liver.

0006-2960/88/0427-5447$01.50/0

that the absence or functional deficiency of one (or possibly more than one) P-450 isozyme may explain the impaired drug biotransformation capacity in PMs (Davies et al., 1981; Meier et al., 1983; Minder et al., 1984; Dayer et al., 1984). The concept that more than one P-450 isozyme may catalyze microsomal metabolism of these substrates has been supported by multiphasic kinetics of some of these reactions (Kronbach et al., 1987; Dayer et al., 1987) and by our recent purification from human liver of two functionally different cytochrome P-450 isozymes both able to catalyze bufuralol 1'-hydroxylation (Gut et al., 1986). Indeed, with (+)- and (−)-bufuralol as substrates, a high-affinity/highly stereoselective isozyme (P450bufI) and a low-affinity/nonstereoselective isozyme (P450bufII) appear to be responsible for bufuralol metabolism in human liver microsomes. It has remained extremely difficult, however, to directly evaluate the relative contribution of these two isozymes or activities in extensive metabolizer (EM) and PM microsomes and to determine if one or both isozymes are affected by the polymorphism.

In the present paper, we describe experiments which allow a differential evaluation of the two catalytic activities in human liver microsomes. We have observed that the purified high-affinity/highly stereoselective isozyme and the corresponding microsomal activity for bufuralol 1'-hydroxylation can be specifically assessed by using the peroxygenase function of this P-450 isozyme [for review of peroxide-supported P-450 reactions, see White and Coon (1980)], whereas the low-affinity/nonstereoselective component is dependent on the classical monooxygenase reaction requiring molecular oxygen, NADPH, and NADPH-cytochrome P-450 reductase (NADPH/O_2 system) for catalysis. The cumene hydroperoxide mediated 1'-hydroxylation of bufuralol was characterized in microsomes of liver samples from extensive and poor metabolizer subjects and in purified P-450 isozymes. These data provide evidence that the debrisoquine/sparteine-type polymorphism of drug oxidation is due to the deficiency of a cytochrome P-450 isozyme with the properties of the recently purified cytochrome P450bufI. Furthermore, we found that antibodies raised against an orthologous P-450 isozyme from male Sprague-Dawley rats (P450db1; Gonzalez et al., 1987) cross-react specifically with P450bufI in human liver microsomes.[2] We demonstrate that the amount of P450bufI protein detected by this antibody correlates with the CuOOH-mediated bufuralol 1'-hydroxylation in microsomes of 14 human livers, including tissue from 8 subjects studied in vivo and in vitro.

Experimental Procedures

Liver Samples and Preparation of Microsomes. Human liver samples were obtained from kidney transplant donors (kidney donor livers, KDL) or as wedge biopsies at laparotomy for diagnostic or therapeutic reasons. Liver pieces were frozen in liquid nitrogen and stored at −80 °C prior to use. All wedge biopsies investigated in this study were from subjects which had been phenotyped in vivo by means of the sparteine or debrisoquine metabolic ratio (Eichelbaum et al., 1979; Price-Evans et al., 1980). The experimental protocol for in vivo testing and use of material from liver biopsies was approved by the ethical review boards (Departments of Medicine, Universities of Zürich and Bonn).

Microsomes from wedge biopsies were prepared according to a micromethod developed in this laboratory (Dayer et al., 1984). Microsomes from kidney donor livers were prepared as follows. The frozen liver was cut into small pieces and thawed rapidly in a beaker containing approximately 4 volumes of 0.15 M KCl. Liver pieces of 1–10 g wet wt were then immediately homogenized in 4 volumes of 1 mM EDTA, 1 mM DL-dithiothreitol, 0.02 mM butylated hydroxytoluene, and 0.1 mM phenylmethanesulfonyl fluoride in 0.15 M KCl with a Polytron 20S homogenizer (Kinematica GmbH, 6010 Kriens, Switzerland) set at 5000 rpm for 10–30 s with constant cooling on ice–water. The homogenate was rehomogenized with three strokes at 800 rpm in a glass–Teflon Potter-Elvehjem homogenizer (pestle–tube clearance 0.18–0.24 mm). A first supernatant was prepared by centrifugation of the homogenate at 12000*g* for 15 min (SS34 rotor, Sorvall). The pellet was discarded and the supernatant recentrifuged at 27000*g* for 15 min. Microsomes were then sedimented from the second supernatant at 105000*g* for 60 min. The pellet was washed once with 1 mM EDTA in 0.1 M sodium pyrophosphate buffer, pH 7.25. After recentrifugation at 105000*g* (60 min), the pellet was rehomogenized with a Dounce homogenizer in 0.1 M sodium phosphate buffer, pH 7.4, to a concentration of approximately 20 mg of protein/mL. All microsomal preparations were kept frozen at −80 °C in small aliquots to avoid repeated freezing.

Assay for Bufuralol 1'-Hydroxylation Activity in Microsomes. Microsomal protein (1–100 μg) was incubated in a final volume of 100 μL of 0.1 M sodium phosphate buffer, pH 7.4, in 1.5-mL plastic tubes. Bufuralol 1'-hydroxylation *mediated by NADPH/O_2* was assayed essentially as described (Kronbach et al., 1987). In brief, the microsomal suspension was preincubated at 37 °C for approximately 5 min before the reaction was started by the addition of 20 μL of a 5-fold concentrated mixture containing a NADPH-regenerating system (final concentration: 1 mM $NADPNa_2$, 5 mM $MgCl_2$, 5 mM isocitrate, 1 unit/mL isocitrate dehydrogenase) and (+)- or (−)-bufuralol (final concentration 0.2 mM except where indicated) in 0.1 M sodium phosphate buffer, pH 7.4. The reaction was stopped after 15 min by the addition of 10 μL of 60% $HClO_4$ (w/v). When the NADPH regenerating system was replaced by *cumene hydroperoxide (CuOOH)*, incubations were performed at 25 °C. CuOOH (80% in cumenol) was diluted first to a concentration of 40 mM in 50% methanol in H_2O (v/v) and then to 1.25 mM in 0.1 M sodium phosphate buffer, pH 7.4; 10 μL of this solution was added to the microsomal suspension together with (+)- or (−)-bufuralol, and the reaction was stopped as described above. Incubation time was between 5 and 10 min.

The production of 1'-hydroxybufuralol was determined in $HClO_4$ supernatants by reversed-phase HPLC with fluorescence detection as previously described (Kronbach et al., 1987). An external standardization procedure with authentic 1'-hydroxybufuralol was applied.

Assay for Debrisoquine 4-Hydroxylation in Microsomes. The CuOOH-mediated 4-hydroxylation of debrisoquine in human liver microsomes was carried out essentially as described for bufuralol 1'-hydroxylation, except that the final assay volume was 150 μL and the substrate concentration was 1 mM. The reaction was stopped after 15 min with 15 μL of 60% $HClO_4$ (w/v). $HClO_4$ supernatants were analyzed for 4-hydroxydebrisoquine by reversed-phase HPLC with fluorescence detection (Kronbach et al., 1987).

Solubilization of Microsomes and Immunoprecipitation Procedures. Human liver microsomes were solubilized at a protein concentration of 3.3 mg/mL in buffer I [20 mM CHAPS, 1 mM EDTA, 1 mM DL-dithiothreitol, and 20% (v/v) glycerol in 0.1 M sodium phosphate buffer, pH 7.4] for

[2] According to the recently proposed nomenclature for P-450 isozymes (Nebert et al., 1987), P450bufI corresponds to P-450 II D1 or P450db1.

1 h at 4 °C. After centrifugation at 105000*g* for 60 min, the supernatant was further diluted 1:5.5 in the same buffer.

Rabbit antibodies were bound to Sepharose beads as follows: 30-μL portions of a 6% (w/v) stock suspension of protein A–Sepharose CL-4B beads (Pharmacia) in 140 mM sodium phosphate buffer (pH 8.0) were pipetted into 1.5-mL Eppendorf tubes and washed once with 1 mL of buffer II (0.2% bovine serum albumin in 0.1 M sodium phosphate buffer, pH 7.4). The pellets were then incubated with appropriate amounts of rabbit antiserum or preimmune serum in a final volume of 0.3 mL of buffer II with rotation end over end at room temperature for 2 h. Control beads were treated identically except no serum was added. After short centrifugation, the supernatant was aspirated and discarded. The pellets were washed twice with 1 mL of buffer II and once with 1 mL of buffer I, before they were incubated for 90 min at 4 °C with 150 μL of solubilized microsomes. The pellets were then spun down, and each supernatant was tested for remaining (+)- or (−)-bufuralol 1′-hydroxylase activity mediated by $NADPH/O_2$ or CuOOH as follows: For determination of $NADPH/O_2$-dependent activity, 12 μL of supernatant was incubated as described above except that cytochrome P-450 reductase (Yasukochi & Masters, 1976) was added to a final concentration of 0.1 μM. At this concentration, P-450 reductase significantly increased the activity but did not change the stereoselectivity or the kinetic properties of CHAPS-solubilized microsomes (data not shown). CuOOH-mediated activity was measured by incubation of 24 μL of supernatant as described above. *Pellets* were washed 3 times with 1 mL of buffer I, washed twice with 1 mL of 0.1 M sodium phosphate buffer, pH 7.4, and resuspended in a final volume of 100 μL of the same buffer prior to determination of CuOOH-mediated activity.

Western Blotting. SDS–PAGE of microsomal protein (50 μg applied per lane) was done in an 8% polyacrylamide gel (Laemmli, 1970), and the proteins were transferred to nitrocellulose according to Towbin et al. (1979). Incubation with anti-rat P450db1 IgG (0.02 mg/mL), visualization of bound antibodies by the peroxidase–antiperoxidase technique, and semiquantitative determination of the stained bands were performed as described previously (Gut et al., 1986).

Analysis of Data and Statistical Methods. Untransformed data from kinetic experiments were analyzed by an iterative nonlinear least-squares fitting program (McIntosh & McIntosh, 1980). Generally, weighting according to the reciprocal of the variance in each point, calculated from at least duplicate incubations, was applied. All results obtained with the CuOOH-mediated reaction as well as the functional characterization of purified cytochrome P450bufI in the reconstituted system were best described on the basis of Michaelis–Menten-type kinetics. For $NADPH/O_2$-dependent microsomal kinetics, the application of a biphasic model with different kinetic parameters for a high- and a low-affinity component resulted in a significant improvement of the curve fits. For the correlations, the nonparametric Spearman rank correlation was used (Snedecor & Cochran, 1980).

Other Methods. The protein content of microsomal preparations was determined by the Lowry method (Lowry et al., 1951) with bovine serum albumin as standard. Cytochrome P-450 content was measured spectrally according to Omura and Sato (1964) on an Aminco-DW2 difference spectrophotometer. NADPH-cytochrome P-450 reductase was purified from untreated rat liver microsomes (Yasukochi & Masters, 1976) and tested for cytochrome *c* reductase activity according to Dignam and Strobel (1978).

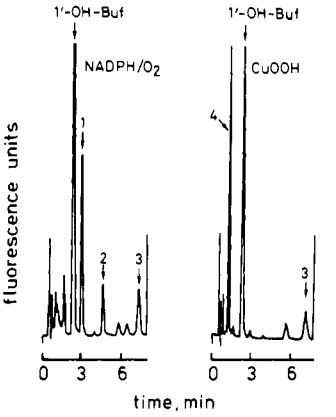

FIGURE 1: HPLC analysis of reaction products of human liver microsomal bufuralol oxidation mediated by $NADPH/O_2$ or cumene hydroperoxide (CuOOH). Human liver microsomes (KDL 19, 19 μg/0.1 mL) were incubated with (+)-burfuralol and a NADPH-regenerating system (37 °C, 15 min) or with 125 μM CuOOH (25 °C, 5 min). Peaks 1–3 indicate metabolites of unknown structure. Their appearance was dependent on the presence of microsomal protein and bufuralol, whereas peak 4 is a reaction product of CuOOH and $HClO_4$. The peak with a retention time of 5.8 min represents an impurity of the substrate.

Table I: Hydroxylation of (+)- and (−)-Bufuralol by Human Liver Microsomes in the Presence of Various Oxygenating Agents

incubation conditions[a]	nmol of 1′-OH-buf mg^{-1} $(60\ min)^{-1}$	(−)/(+) ratio[b]
$NADPH/O_2$	42.9 (37 °C)	0.46
	8.8 (25 °C)	0.47
CuOOH (125 μM)	92.9 (25 °C)	0.28
iodosobenzene (200 μM)	82.7 (25 °C)	0.65
tert-butyl hydroperoxide (2 mM)	43.9 (25 °C)	0.25
H_2O_2 (1 mM)	0.3 (37 °C)	

[a] Human liver microsomes (KDL 19, 19 μg/0.1 mL) were incubated with either (+)-bufuralol in the presence of NADPH or different oxygenating agents at the temperatures indicated. Incubatioon time was 1 min for iodosobenzene and *tert*-butyl hydroperoxide and 15 min for hydrogen peroxide. Results are means of two incubations. [b] Ratio of activities for (−)- and (+)-bufuralol 1′-hydroxylation.

Materials. (+)- and (−)-bufuralol, 1′-hydroxybufuralol, and debrisoquine were gifts from Hoffmann-La Roche, Basel, Switzerland, and Welwyn-Garden City, U.K. Goat antirabbit IgG (GaR/7S) and peroxidase–antiperoxidase complex (R/PAP) were from Nordic Immunological Laboratories, Tilburg, The Netherlands. Iodosobenzene was kindly provided by Dr. H. Graf, Konstanz, FRG. Cumene hydroperoxide (80% in cumenol) was from Merck, Darmstadt, FRG. All other chemicals were from Sigma, St. Louis, MO, or from Merck, Darmstadt, FRG.

RESULTS

Characterization of CuOOH-Mediated Bufuralol Hydroxylation in Human Liver Microsomes and Purified P450bufI and P450bufII

Metabolite Pattern and Stereoselectivity of Microsomal Bufuralol Metabolism. Incubation of human liver microsomes in the presence of bufuralol and CuOOH resulted in the formation of 1′-hydroxybufuralol as the major reaction product detectable by fluorescence HPLC (Figure 1). One additional fluorescent metabolite with a longer retention time than 1′-hydroxybufuralol was observed. The formation of 1′-hydroxybufuralol displayed a remarkable stereoselectivity for the (+) enantiomer of bufuralol with a (−)/(+) ratio of 0.28 (Table I). In contrast, incubation of the same microsomal preparation with an NADPH-regenerating system (designed

in this paper as NADPH/O_2 system) catalyzed the formation of at least two additional unidentified metabolites (Figure 1). These metabolites were exclusively observed with reconstituted P450bufII but not with reconstituted P450bufI (Gut et al., 1986). Moreover, the reaction with NADPH/O_2 was remarkably less stereoselective for (+)-bufuralol [(−)/(+) ratio of 0.46; Table I].

Optimal Conditions for CuOOH-Mediated Bufuralol 1′-Hydroxylation. The rate of formation of 1′-hydroxybufuralol was maximal at around 150 μM CuOOH and was linear with time for at least 10 min at a concentration of 125 μM CuOOH and at 25 °C (data not shown). Under these conditions the pH dependence of the reaction was characterized by a broad maximum with optimal rate between pH 7.0 and pH 9.0 (data not shown). For convenience, standard incubations were performed at pH 7.4, which represents the optimum for NADPH/O_2-dependent bufuralol 1′-hydroxylation. Formation of 1′-hydroxybufuralol was linear with protein from 0.01 to at least 1 mg/mL.

At higher CuOOH concentrations and after longer incubation times, the reaction rate decreased, presumably due to destruction of microsomal P-450 by the organic hydroperoxide (Capdevila et al., 1980). When microsomes were incubated at 37 °C, product formation was linear for less than 2 min (data not shown).

Effect of Other Oxygenating Agents and of Inhibitors. In addition to CuOOH, iodosobenzene and *tert*-butyl hydroperoxide also supported the formation of 1′-hydroxybufuralol whereas hydrogen peroxide had no effect (Table I). A decreased stereoselectivity was observed with iodosobenzene as compared to CuOOH or *tert*-butyl hydroperoxide. The reason for this difference is unknown, it may reflect a different reaction mechanism [p 232 of Ortiz de Montellano (1986)] or a different selectivity of iodosobenzene for P-450 isozymes. Common inhibitors of P-450 function like KCN (5 mM), metyrapone (250 μM), or SKF 525 A (50 μM) decreased microsomal CuOOH-mediated bufuralol 1′-hydroxylation by more than 90%. Carbon monoxide, as expected, inhibited only the NADPH/O_2-dependent monooxygenase reaction. Superoxide dismutase (500 units/mL), catalase (500 units/mL), or cumenol (0.1 mM), which is present in the used CuOOH preparation as a solvent, had no influence on the activity (data not shown).

Effect of Substrate Concentration on Microsomal Bufuralol 1′-Hydroxylation Mediated by NADPH/O_2 versus CuOOH. The initial experiments (Figure 1 and Table I) revealed differences in regard to the stereoselectivity and the metabolite pattern between the reactions supported by NADPH/O_2 and CuOOH. As described recently (Dayer et al., 1987; Kronbach et al., 1987), detailed kinetic analyses of the NADPH/O_2-mediated bufuralol 1′-hydroxylation in human liver microsomes consistently resulted in multiphasic reactions, suggesting the participation of at least two enzymes.

In striking contrast we found the CuOOH-mediated reaction being monophasic in all livers investigated so far. A typical Eadie–Hofstee plot of (+)-bufuralol 1′-hydroxylation is shown in Figure 2. Similar results were observed with (−)-bufuralol as substrate (data not shown).

In the NADPH/O_2 system, the analysis of these data indicated an about equal contribution to the total activity by a high-affinity component (K_m = 5.3 μM) with high stereoselectivity and a low-affinity component (K_m = 121 μM) with low stereoselectivity (Table II). The apparent K_m and the stereoselectivity of the monophasic reaction supported by CuOOH closely resembled those of the high-affinity component of the NADPH/O_2 system. It has to be noted, however, that the V_{max} values of these two systems are not directly comparable because the incubations are done at different temperatures.

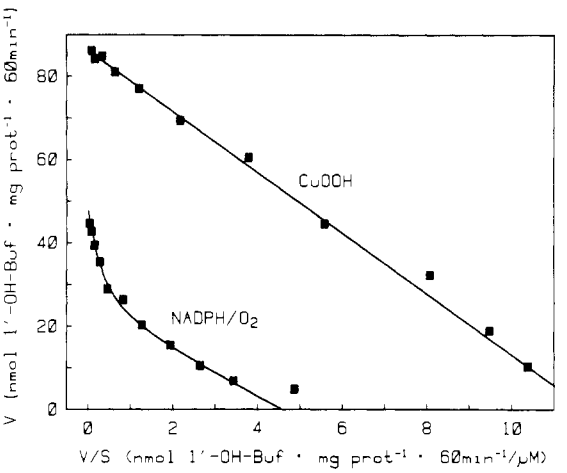

FIGURE 2: Kinetic analysis (Eadie–Hofstee plot) of microsomal (+)-bufuralol 1′-hydroxylation mediated by NADPH/O_2 or CuOOH. The rate of NADPH/O_2- and CuOOH-supported (+)-bufuralol 1′-hydroxylation (V) was measured as a function of (+)-bufuralol concentration (S) in a range of 1–1000 μM. Each point is the mean of two incubations. A typical example for microsomes with high bufuralol 1′-hydroxylase activity is shown (KDL 19). The untransformed results were analyzed with the fitting program described under Experimental Procedures, and the lines were drawn from the K_m and V_{max} parameters listed in Table II.

Table II: Comparison of Kinetic Parameters of Microsomal Bufuralol 1′-Hydroxylation Mediated by NADPH/O_2 or CuOOH[a]

assay system	K_m ± SE[b]	V_{max} ± SE[b]	(−)/(+) ratio[c]
NADPH/O_2			
enzyme 1	5.3 ± 0.4	23.0 ± 1.2	0.26
enzyme 2	121.0 ± 22.0	24.7 ± 1.0	0.61
CuOOH	7.3 ± 0.1	86.2 ± 0.3	0.29

[a] The untransformed results of Figure 2 were analyzed with a nonlinear least-squares fitting program. Michaelis–Menten-type kinetics offered an adequate description of the results for the CuOOH-mediated reaction. For the NADPH/O_2-dependent reaction the data are best described with a two-enzyme model (see text). [b] K_m (μM) and V_{max} [nmol of 1′-hydroxybufuralol mg^{-1} (60 $min)^{-1}$] are those for (+)-bufuralol as substrate. SE is standard error of K_m and V_{max}. [c] The (−)/(+) ratio was calculated as V_{max} (−)-bufuralol)/V_{max}((+)-bufuralol).

Correlation between CuOOH-Supported Bufuralol 1′-Hydroxylation and Debrisoquine 4-Hydroxylation. Because bufuralol 1′-hydroxylation is one of the prototype reactions for the debrisoquine/sparteine-type polymorphism of drug oxidation, we tested whether CuOOH also supports debrisoquine 4-hydroxylation. Human liver microsomes indeed catalyzed the CuOOH-mediated 4-hydroxylation of debrisoquine in a protein- and time-dependent manner. We measured both activities in liver microsomes of 19 organ donors representing a wide range of activities (Figure 3). The close correlation between these two activities (r_s = 0.963, $P < 0.001$) strongly suggests that the same P-450 isozyme (or isozymes under closely linked control) is responsible for both reactions.

It was thus reasonable to assume that the CuOOH-mediated bufuralol 1′-hydroxylation could be catalyzed selectively by the high-affinity/highly stereoselective component in human liver microsomes. The following experiments were designed to proof this hypothesis.

Inhibition by Quinidine of Microsomal Bufuralol 1′-Hydroxylation. Quinidine has been shown to be a potent inhibitor in vivo and in vitro of substrate hydroxylations affected by the debrisoquine/sparteine-type polymorphism

Table III: Kinetic Parameters of Bufuralol 1'-Hydroxylation in Microsomes and Purified Cytochromes P450bufI and P450bufII

	assay system	K_m (μM) [with (+)-bufuralol]	V_{max} [nmol mg^{-1} (60 min)$^{-1}$]	(−)/(+) ratio	K_i of quinidine (μM)
microsomes[a]	CuOOH	36 ± 35	48 ± 33	0.34 ± 0.07	0.15 ± 0.04[b]
P450bufI[c]	NADPH/O_2	16.8	3980	0.17	0.027
	CuOOH	20.6	2650	0.29	0.40
P450bufII[d]	NADPH/O_2	245	776	1.03	80
	CuOOH	nd[e]	nd	nd	nd

[a] Means ± standard deviation for K_m, V_{max} [with (+)-bufuralol as substrate], and (−)/(+) ratio were calculated from the kinetic parameters of microsomes of extensive metabolizers shown in Figure 5 (n = 11). [b] Determined in microsomes of three livers with high bufuralol 1'-hydroxylase activity (KDL 19, 23, and 27). [c] Cytochrome P450bufI was purified from KDL 23. For assay of bufuralol 1'-hydroxylation, 0.8 pmol of P450bufI (2.56 nmol of cytochrome P-450/mg of protein) was incubated as described in the text. [d] Cytochrome P450bufII was purified from KDL 16. The data for NADPH/O_2 are from Gut et al. (1986). [e] nd, below the limit of detection with 10 pmol of P450bufII/0.1 mL of sodium phosphate buffer, pH 7.4.

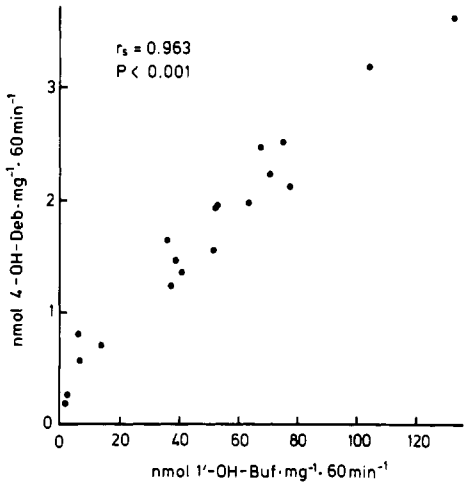

FIGURE 3: Relationship between CuOOH-mediated debrisoquine 4-hydroxylation and bufuralol 1'-hydroxylation in microsomes of 19 human livers. Activities were determined at 25 °C and with 100 μg of microsomal protein. Results are means of two incubations. The correlation coefficient r_s was calculated from the nonparametric Spearman rank correlation.

(Otton et al., 1984; Leemann et al., 1986). Moreover, we have recently demonstrated a strong inhibition by quinidine of bufuralol 1'-hydroxylation with the reconstituted high-affinity/highly stereoselective isozyme P450bufI, whereas with the low-affinity/nonstereoselective P450bufII a more than 1000-fold lower inhibitory potency was observed (Gut et al., 1986).

We therefore compared the inhibition by quinidine of both the microsomal CuOOH-mediated and microsomal NADPH/O_2-mediated bufuralol 1'-hydroxylation over a wide range of inhibitor concentrations (Figure 4). Whereas the CuOOH-mediated 1'-hydroxylations of (+)- and (−)-bufuralol were almost completely inhibited (Figure 4A), with NADPH/O_2 a considerable part of the total activity was less sensitive to the inhibitor, and the increasing difference between (−)- and (+)-bufuralol 1'-hydroxylation at higher quinidine concentrations indicated a lower stereoselectivity of this remaining activity (Figure 4B). Inhibition data at different bufuralol concentrations (not shown) were subjected to kinetic analysis using a nonlinear least-squares fitting program. The results obtained from three different livers with high bufuralol 1'-hydroxylase activity revealed competitive inhibition at a single site for the CuOOH-mediated reaction (K_i = 150 ± 40 nM, Table III). The data obtained with the NADPH/O_2 system were in agreement with a model assuming two enzymes both inhibited competitively by quinidine but with a much lower potency for the high-K_m component.

CuOOH- and NADPH/O_2-Mediated Bufuralol 1'-Hydroxylation in Purified P450bufI and P450bufII. In order to purify the P-450 isozyme responsible for the microsomal

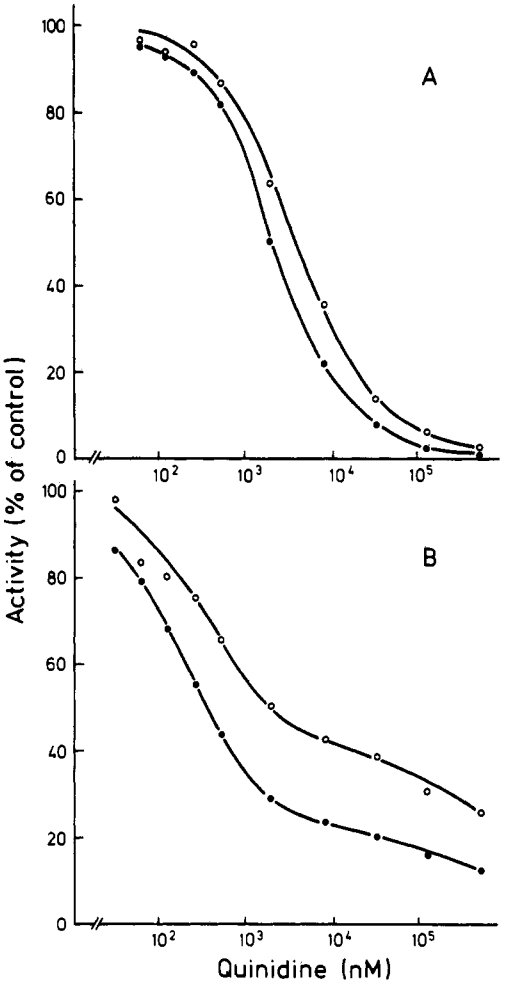

FIGURE 4: Inhibition by quinidine of microsomal (+)- and (−)-bufuralol 1'-hydroxylation. Bufuralol 1'-hydroxylation in human liver microsomes (KDL 19) was supported either by CuOOH (A) or by an NADPH-regenerating system (B), and the effect of quinidine was compared in the two systems. Closed symbols represent measurements of (+)-bufuralol 1'-hydroxylation in percent of control, open symbols refer to (−)-bufuralol. Substrate concentration was 100 μM for (+)- and (−)-bufuralol. Control activity (100%) was 91.3 and 23.9 (A) or 39.8 and 18.3 (B) nmol of 1'-OH-buf mg^{-1} (60 min)$^{-1}$ for (+)- and (−)-bufuralol, respectively. Each point represents the mean of two incubations. The kinetic analysis of the data is described in the text.

CuOOH-mediated bufuralol 1'-hydroxylation, we used this reaction as a screening assay for column eluates in a purification scheme[3] similar to the procedure applied earlier in the purification of cytochrome P450bufI (Gut et al., 1986). The

[3] Francis Vilbois, Ulrich, M. Zanger, and Urs A. Meyer, unpublished results.

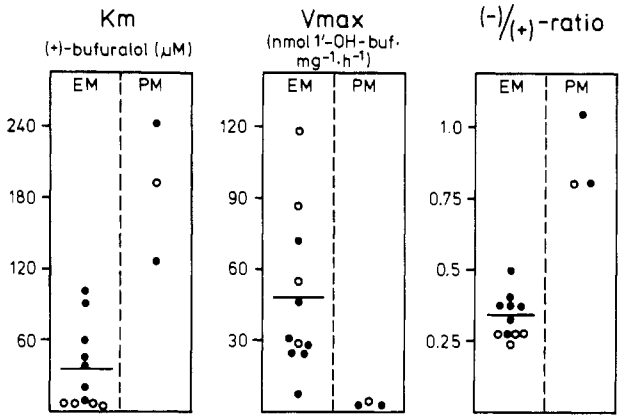

FIGURE 5: CuOOH-mediated (+)-bufuralol 1'-hydroxylation in microsomes from extensive (EM) and poor metabolizer (PM) subjects. Nine liver samples were wedge biopsies from subjects phenotyped in vivo [(●) seven EM and two PM]; the other five samples (○) were studied in vitro only. Untransformed kinetic data were analyzed by the described curve fitting program. The (-)/(+) ratio was determined at 500 μM (+)- and (-)-bufuralol.

functional characterization of the obtained purified fraction under the described reconstitution conditions revealed the highest specific activity for bufuralol 1'-hydroxylation reported so far (Gut et al., 1986; Distlerath et al., 1985) with negligible production of additional metabolites, a high stereoselectivity for (+)-bufuralol, and competitive inhibition by quinidine with a K_i in the nanomolar range (Table III). This purified enzyme thus has the same characteristics as P450bufI. It was highly active as a peroxygenase, and the kinetic parameters of CuOOH-mediated bufuralol 1'-hydroxylation closely resembled those of the microsomal reaction (Table III). In contrast, cytochrome P450bufII, prepared as previously described (Gut et al., 1986), was only active in bufuralol 1'-hydroxylation as a monooxygenase, and no activity could be detected in repeated incubations with CuOOH.

P450bufI Is the Target of the Debrisoquine/Sparteine-Type Polymorphism of Drug Oxidation

Kinetic Analysis of CuOOH-Supported Bufuralol 1'-Hydroxylation in Microsomes of in Vivo Phenotyped Human Liver Biopsies. To evaluate the role of P450bufI in the debrisoquine/sparteine-type polymorphism, we next studied the CuOOH-mediated 1'-hydroxylation of bufuralol in microsomes prepared from liver biopsies of in vivo phenotyped EM and PM individuals. A striking selectivity of the CuOOH-mediated reaction in identifying the PM condition was observed (Figure 5). Drastically reduced V_{max} and increased K_m of the low remaining activity characterized the PM samples. Moreover, the high stereoselectivity of the CuOOH-supported bufuralol 1'-hydroxylation observed with EM microsomes was almost completely lost in the poor metabolizers. This deficiency of PM microsomes in P450bufI-type activity was further substantiated by a more than 1000-fold lower inhibitory potency of quinidine for the activity of PM as compared to EM microsomes (data not shown).

Cross-Reactivity and Specificity of Anti-Rat P450db1 in Human Liver Microsomes. Recently, we have developed a rabbit antiserum against purified P450db1, a P-450 isozyme from male Sprague-Dawley rats with high specific activity for debrisoquine 4-hydroxylation and bufuralol 1'-hydroxylation (Gonzalez et al., 1987). To test the possibility that this antibody cross-reacts with bufuralol-metabolizing P-450 isozymes in human liver, we performed immunoprecipitation and immunoinhibition studies.

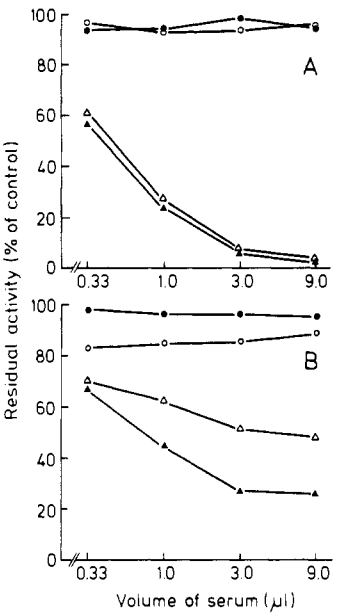

FIGURE 6: Immunoprecipitation of bufuralol 1'-hydroxylase activity from solubilized microsomes. Solubilized human liver microsomes (KDL 19) were incubated with increasing amounts of rabbit anti-rat P450db1 IgG bound to protein A–Sepharose. After centrifugation supernatants were tested for residual (+)- and (-)-bufuralol 1'-hydroxylation activity, mediated by CuOOH (A) or NADPH/O_2 (B). Anti-rat P450db1 serum: (▲) (+)-bufuralol; (△) (-)-bufuralol. Preimmune serum: (●) (+)-bufuralol; (○) (-)-bufuralol. Control activity (100%, no serum added) was 24.8 and 6.5 (A) or 11.6 and 5.0 (B) nmol of 1'-OH-buf mg^{-1} (60 $min)^{-1}$ for (+)- and (-)-bufuralol, respectively. Each point reflects the mean of two immunoprecipitations.

As shown in Figure 6, anti-rat P450db1 immunoprecipitated specifically P450bufI-type activity from solubilized human liver microsomes. This is evident from the complete precipitation of CuOOH-mediated (+)- and (-)-bufuralol 1'-hydroxylase activity (Figure 6A) and the only partial precipitation of the NADPH/O_2-dependent activity (Figure 6B). The residual activity detected with NADPH/O_2 displayed almost no stereoselectivity but retained its ability to produce other metabolites in addition to 1'-hydroxybufuralol as previously observed with this assay system for purified P450bufII (Gut et al., 1986; Figure 1). Bufuralol 1'-hydroxylase activity was also detectable in the precipitates where it increased reciprocally with the disappearance of activity from the supernatants (data not shown).

Direct inhibition by anti-rat P450db1 of the NADPH/O_2-mediated activity in intact microsomes resulted in 73% inhibition (200 μL of antiserum/nmol of P-450) with a similar decrease in stereoselectivity of the remaining activity as revealed by the immunoprecipitation experiment (data not shown).

Correlation between CuOOH-Mediated Bufuralol 1'-Hydroxylation and Western Blots with Anti-Rat P450db1 in Human Liver Microsomes. On Western blots of human liver microsomes of extensive metabolizers as well as purified cytochrome P450bufI, anti-rat P450db1 IgG reacted with a protein of approximately M_r 50K (Figure 7). No immunoreactive material of the same molecular weight could be detected in microsomes of three poor metabolizers including tissue from two subjects phenotyped in vivo. Moreover, the relative density of the stained bands in microsomes of 14 human livers (6 kidney organ donor livers, 8 wedge biopsies from individuals phenotyped in vivo) correlated strongly (r_s = 0.95, $P < 0.001$) with the respective V_{max} values for CuOOH-mediated bufuralol 1'-hydroxylation (Figure 7).

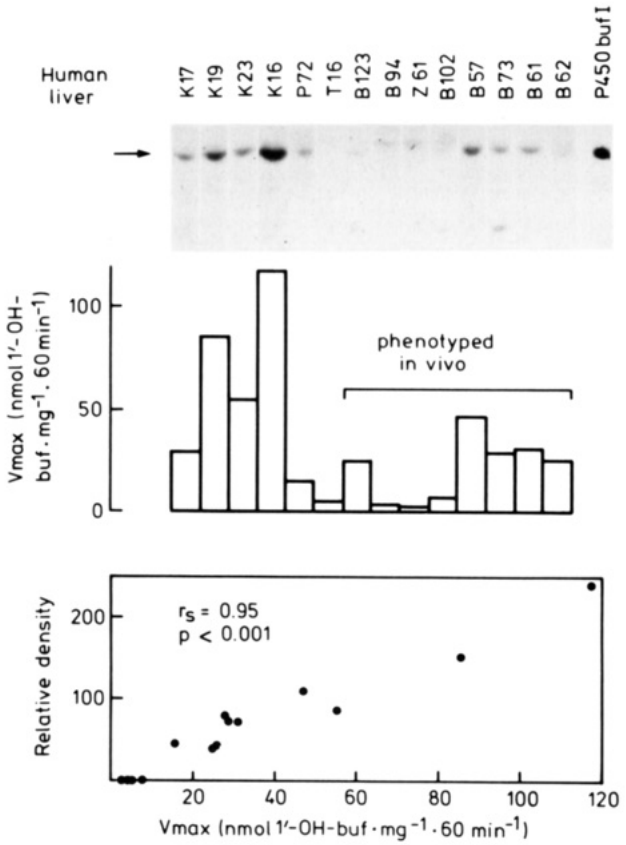

FIGURE 7: Relationship between CuOOH-mediated bufuralol 1′-hydroxylation and immunoblots with anti-rat P450db1 IgG in microsomes of 14 human livers. 50 μg of microsomal protein from each of 14 human livers, including 8 wedge biopsies from in vivo phenotyped individuals and 6 KDL samples from in vitro characterized tissues (K17, K19, K23, K16, P72, and T16), as well as 12 μg of purified P450bufI was subjected to SDS–PAGE in an 8% polyacrylamide gel, and the proteins were transferred to nitrocellulose. The nitrocellulose sheet was then probed with anti-rat P450db1 IgG. The immunochemical reaction on each lane at M_r 50K (→) was semiquantitatively determined with a CAMAG high-performance gel scanner in the reflection mode, and the results were compared with the respective V_{max} values for CuOOH-mediated bufuralol 1′-hydroxylation by means of the Spearman rank correlation. The urinary metabolic ratios (MR) of the eight in vivo phenotyped subjects were determined with sparteine (S) (Eichelbaum et al., 1979) or debrisoquine (D) (Price-Evans et al., 1980). Two patients were PMs [B94, MR (S) 45.0; Z61, MR (D) 66.0]; four patients were EMs [B123, MR (S) 0.2; B57, MR (S) 0.18; B73, MR (S) 0.36; B61, MR (S) 0.47]. B102 and B62 had intermediate MRs (S) of 3.0 and 1.9, respectively.

Determination of the NADPH/O_2-mediated bufuralol 1′-hydroxylation in these liver microsomes revealed less pronounced differences between EM and PM samples and suggested the presence of nonstereoselective P450bufII-type activity in PMs (Dayer et al., 1987). Hence, the relationship between the immunoreaction and the NADPH/O_2-mediated activity was less clear, yet still significant ($r_s = 0.87$, $P < 0.001$, data not shown).

Discussion

The data presented here provide evidence that the debrisoquine/sparteine-type genetic polymorphism of drug oxidation in man is caused by the complete or almost complete absence of a P-450 isozyme with the characteristics of P450bufI, previously purified from human liver microsomes in this laboratory (Gut et al., 1986). This conclusion is derived from the following observations: (1) The cumene hydroperoxide (CuOOH) mediated 1′-hydroxylation of bufuralol was exclusively catalyzed by the P450bufI isozyme, and in microsomes of in vivo phenotyped poor metabolizers of debrisoquine or sparteine, this activity was drastically reduced. (2) Polyclonal antibodies specifically recognizing P450bufI showed no immunoreactivity with microsomes of poor metabolizers.

In earlier studies in this and other laboratories the P-450 activities affected by the debrisoquine/sparteine-type polymorphism in microsomes (Dayer et al., 1987; Eichelbaum et al., 1986; Boobis et al., 1985) and purified P-450 isozymes (Gut et al., 1986; Distlerath et al., 1985) were analyzed as classical monooxygenase reactions dependent on molecular oxygen, NADPH, and NADPH-cytochrome P-450 reductase. In this assay system and with bufuralol as substrate at least two functionally distinct P-450 isozymes contribute to microsomal bufuralol 1′-hydroxylation, namely, P450bufI with high affinity and high stereoselectivity for (+)-bufuralol and P450bufII with low affinity and no stereoselectivity (Gut et al., 1986). The contribution of more than one isozyme to the overall activity in microsomes may explain, for instance, why studies with antibodies raised against a rat P-450 isozyme with high debrisoquine 4-hydroxylase activity showed only a limited ($r_s = 0.57$) relationship between an immunoreactive human microsomal protein and NADPH/O_2-dependent debrisoquine 4-hydroxylation activity (Distlerath & Guengerich, 1984). The lack of isozyme-specific assays and of isozyme-specific antibodies so far precluded a differential evaluation of these two isozymes as possible targets of the genetic defect.

The following data support the assumption that CuOOH-mediated bufuralol 1′-hydroxylation is catalyzed exclusively or almost exclusively by P450bufI: (a) The reaction was monophasic in all EM microsomes tested whereas the NADPH/O_2-supported bufuralol 1′-hydroxylation consistently was multiphasic. (b) Inhibition by quinidine, previously shown to be a potent competitive inhibitor of P450bufI and which has little affinity for P450bufII (Gut et al., 1986), completely inhibited the CuOOH-mediated but only partially the NADPH/O_2-mediated reaction. (c) The purified human P450bufI fraction had a high turnover number in the CuOOH system whereas P450bufII had no detectable activity.

A combined analysis of immunoprecipitation and immunoinhibition experiments with the rabbit antiserum selected for these studies and with both the CuOOH and NADPH/O_2 assay systems indicated that anti-rat P450db1 specifically recognizes P450bufI in human liver microsomes (Figure 6). Moreover, Western blots of EM microsomes with these antibodies revealed a close correlation between the immunoreactive protein and the CuOOH-mediated bufuralol 1′-hydroxylation (Figure 7), providing further evidence that both the CuOOH-mediated reaction and the antibody identify the same P-450 isozyme, P450bufI.

When these new and more specific tools were applied to investigate the role of P450bufI in the debrisoquine/sparteine-type polymorphism, we observed a striking decrease in the V_{max} of P450bufI-type activity and the absence of immunoreactive protein in microsomes of two subjects characterized in vivo as poor metabolizers. We therefore propose that this polymorphism is the result of the complete or almost complete deficiency of P450bufI.[2] This deficiency most probably is caused by decreased enzyme protein, due to deficient synthesis or instability or, less likely, due to a major change in structure, leading both to nonrecognition by the polyclonal antibody and to loss of activity.

Added in Proof

Since submission of the manuscript, aberrant splicing of pre-mRNA of P450db1 (which is identical with P450bufI)

has been observed in two livers with low activity of bufuralol 1'-hydroxylase (Gonzalez et al., 1988). This probably results in the formation of unstable proteins and offers an explanation for the absence of P450bufI/db1 described here.

ACKNOWLEDGMENTS

We thank Dr. Dieter Walz (Biocenter of the University of Basel) for expert help with computer modeling of kinetic data and Dr. Thomas Kronbach (Biocenter of the University of Basel) for many helpful discussions as well as Therese Catin for technical assistance. Philippe Beaune (Paris), Werner Kalow, and Ted Inaba (Toronto) are gratefully acknowledged for supplying liver samples KDL P72 and KDL T16, respectively. Marianne Liechti provided excellent secretarial assistance.

Registry No. P-450, 9035-51-2; buf, 54340-62-4; (−)-buf, 64100-62-5; (+)-buf, 64100-61-4; 1'-OH-buf, 57704-16-2; sparteine, 90-39-1; debrisoquine, 1131-64-2.

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