Pergamon

Biochemical Pharmacology, Vol. 47, No. 11, pp. 1957–1963, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0119-6

DIFFERENTIAL ROLES OF CYTOCHROMES P450 2D1, 2C11, AND 1A1/2 IN THE HYDROXYLATION OF BUFURALOL BY RAT LIVER MICROSOMES

MAYUMI MIMURA,* HIROSHI YAMAZAKI,* CHIKAKO SUGAHARA,* TOYOKO HIROI,† YOSHIHIKO FUNAE† and TSUTOMU SHIMADA*‡

*Osaka Prefectural Institute of Public Health, Osaka 537, Japan; and †Osaka City University School of Medicine, Osaka 545, Japan

(*Received* 7 *December* 1993; *accepted* 3 *February* 1994)

Abstract—Bufuralol hydroxylation activities of liver microsomal cytochrome P450 (P450) enzymes were studied in the rat; the reaction has been used widely in determining levels of liver microsomal P450 2D6, which shows debrisoquine-type genetic polymorphism in humans. Liver microsomes catalyzed the conversion of bufuralol to 1′-hydroxybufuralol and a structurally unidentified metabolite (termed here as M-1) in the presence of an NADPH-generating system and molecular oxygen. Bufuralol 1′-hydroxylation activities catalyzed by the liver microsomes were not increased in rats treated with several P450 inducers, whereas β-naphthoflavone treatment (and to a lesser extent that of isosafrole) caused a significant induction of M-1 formation. The major role of P450 1A1/2 in M-1 formation was confirmed by catalytic inhibition with anti-P450 antibodies and α-naphthoflavone in liver microsomes of β-naphthoflavone-treated rats, and by reconstitution experiments containing P450 1A1 and 1A2. Among nine forms of purified rat P450 enzymes studied in the reconstituted system, P450 2C11 displayed the highest activities for bufuralol 1′-hydroxylation, followed by P450 1A1 and P450 2D1. A female-specific form of P450 2C12 did not catalyze bufuralol 1′-hydroxylation. In liver microsomes of male rats, however, P450 2D1 was the dominant enzyme because only anti-P450 2D1 antibodies, and not anti-P450 2C11 and anti-P450 1A1, inhibited the bufuralol hydroxylation activities, and a specific P450 2D1 inhibitor, quinine, caused a dramatic decrease in the hydroxylation activities. The major contribution of P450 2D1 in the bufuralol 1′-hydroxylation activities was also supported by a kinetic analysis of the reconstituted system; P450 2D1 enzyme had a very low K_m value (8.4 μM) as compared with those of P450 2C11 (K_m = 83 μM) and P450 1A1 (K_m = 230 μM). Thus, the present results suggested that different P450 enzymes are involved in the hydroxylation of bufuralol in rat liver microsomes, and the kinetic analysis, as well as immunoinhibition and chemical inhibition experiments, may be of great importance for determining the major roles of P450 enzymes in drug hydroxylation reactions.

Key words: P450 2D1; bufuralol; P450 1A1; P450 1A2; P450 2C11; kinetic analysis

Bufuralol, a β-adrenoreceptor antagonist, has been used widely as a good substrate for determining the levels of liver microsomal P450 2D proteins, enzymes known to show debrisoquine/sparteine-type genetic polymorphism in humans [1–3]. This drug is metabolized by P450 enzymes, resulting in the formation of 1′-hydroxybufuralol and several structurally unidentified metabolites; the former metabolite has been reported to be catalyzed mainly by P450 2D enzymes in rat and human liver microsomes [2, 4–6]. However, the possible roles of other P450s in the hydroxylation activities of bufuralol by rat and human liver microsomes have not been characterized as extensively as those of P450 2D enzymes [5, 7–9]. In 1984, Larrey *et al.* [10] reported that, among several rat P450 enzymes examined, $P450_{UT\text{-}A}$ (now identified as P450 2C11) and $P450_{UT\text{-}H}$ (now identified as P450 2D1) catalyze debrisoquine 4-hydroxylation in the reconstituted monooxygenase system, although only anti-$P450_{UT\text{-}H}$ is effective in inhibiting debrisoquine 4-hydroxylation catalyzed by liver microsomes of Sprague–Dawley rats. Also of interest is the finding that liver microsomes isolated from the female DA rat, a strain deficient in the expression of P450 2D1, can catalyze bufuralol 1′-hydroxylation, suggesting that other forms of P450 may also participate in the reaction [10, 11].

This study, therefore, was undertaken to determine the possible roles of rat P450§ enzymes in the hydroxylation of bufuralol, using liver microsomes of rats treated with various P450 inducers and a reconstituted monooxygenase system containing nine forms of purified rat P450 enzymes. We also used chemical P450 inhibitors, including SKF-525A, metyrapone, ANF, quinine, quinidine, methylpyrazole, and gestodene and antibodies raised against several rat P450 enzymes, to identify which P450 forms are principal enzymes in catalyzing bufuralol hydroxylation. Upon analysis with high-performance liquid chromatography, we detected at least two hydroxylated metabolites, namely 1′-

‡ Corresponding author: Dr. T. Shimada, Osaka Prefectural Institute of Public Health, 3–69 Nakamichi 1-chome, Higashinari-ku, Osaka 537, Japan. Tel. 81-6-972-1321; FAX 81-6-972-2393.

§ Abbreviations: P450, liver microsomal cytochrome P450; PB, phenobarbital; BNF, β-naphthoflavone; ISF, isosafrole; DEX, dexamethasone; ANF, α-naphthoflavone; and SKF-525A, β-diethylaminoethyl diphenylpropylacetate.

hydroxybufuralol and a structurally unidentified metabolite (termed M-1), after incubating racemic bufuralol with rat liver microsomes. An interesting finding was that different P450 enzymes may be involved in the formation of these two metabolites by rat liver microsomes. The role of P450 enzymes in bufuralol hydroxylation activities was also determined by kinetic analysis in a reconstituted monooxygenase system, and the results obtained suggest that kinetic analysis, as well as immunoinhibition and chemical inhibition experiments, is of great importance for identifying the major roles of P450 enzymes in hydroxylation reactions.

MATERIALS AND METHODS

Chemicals. (±)-Bufuralol and 1′-hydroxybufuralol were donated by Dr. F. Peter Guengerich, Vanderbilt University School of Medicine. Other chemicals that were used were from the same sources described previously [12–14].

Enzyme preparations and antibodies. Male and female Sprague–Dawley rats (weighing about 200 g) were obtained from the Nihon Clea Co., Osaka. In some experiments, male rats were treated i.p. with PB (80 mg/kg, daily for 3 days), BNF (50 mg/kg, daily for 3 days), ISF (100 mg/kg, daily for 3 days) or DEX (50 mg/kg daily for 4 days) and were starved overnight before being killed. Liver microsomes were prepared as described previously [15] and suspended in 10 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM EDTA and 20% glycerol (v/v).

Rat P450 1A1, 1A2, 2B1, 2B2, 2C6, 2C11, 2C12, 2D1, and 3A2 were purified to electrophoretic homogeneity as described previously [13, 16–18]. Rabbit anti-P450 antibodies were prepared [16, 17, 19, 20] and IgG fractions were obtained using a single DEAE-cellulose column step [21]. Rabbit liver NADPH–P450 reductase and cytochrome b_5 were purified by the method of Yasukochi and Masters [22], as modified by Taniguchi *et al.* [23].

Assay methods. Bufuralol 1′-hydroxylase activities by liver microsomal P450 enzymes were determined according to the method of Kronbach *et al.* [6, 24] with slight modification. The incubation mixture consisted of rat liver microsomes (1 mg protein/mL) and bufuralol (final concentration 0.026 to 0.4 mM) in a final volume of 0.1 mL of 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system, as described previously [12]. The reconstituted P450 system was composed of 10–25 pmol of purified rat P450, 20–50 pmol of cytochrome b_5, 50 pmol of NADPH–P450 reductase, and 10 μg of a phospholipid mixture consisting of L - α - dilauroyl - *syn* - glycero - 3 - phosphocholine, dioleoylphosphatidylcholine and L-α-phosphatidyl-L-serine (1:1:1) per mL and 100 μg of sodium cholate/mL [25]. Incubations were carried out at 37°C for 30 min, and terminated by adding 10 μL of 60% perchloric acid. The mixtures were centrifuged at 3000 g for 5 min, and aliquots of the supernatant were injected onto columns for the determination of hydroxylated metabolites by high-performance liquid chromatography (model CCPD, Tosoh Co., Tokyo). The separation was carried out with an octyldecylsilyl reverse-phase column (4.6 × 250 mm; Chromato Tec Co., Tokyo) eluted with a mixture of 45% acetonitrile containing 20 mM sodium perchlorate (pH 2.5). The hydroxylated metabolites of bufuralol were detected by a fluorescence detector (model RF-530, Shimadzu, Kyoto). The retention times for the hydroxylated metabolites in this assay were as follows: 1′-hydroxybufuralol (3.0 min), unidentified metabolite M-1 (3.5 min), and bufuralol (5.7 min). It should be mentioned that Kronbach *et al.* [6] have detected three minor metabolites, as well as 1′-hydroxybufuralol, upon incubating bufuralol with human liver microsomes; they proposed, upon analysis with combined gas chromatography–mass spectrometry, that one of the metabolites may be a hydroxylation product of the ethyl-coumaron part of bufuralol. Although attempts were not made in this study to identify the structure of M-1, its formation was suggested to be catalyzed by rat P450 enzymes, based on the results of the effects of several chemical P450 inhibitors and anti-P450 antibodies (*vide infra*). In this study, therefore, we tentatively represent the activities for formation of M-1 on the basis of a chromatographic response using 1′-hydroxybufuralol as a standard.

Other assays. P450 and protein contents were estimated spectrally by the methods of Omura and Sato [26] and Lowry *et al.* [27], respectively.

Statistical analysis. Statistical analysis was determined by Student's *t*-test.

RESULTS AND DISCUSSION

(±) Bufuralol hydroxylation activities in rat liver microsomes. Racemic bufuralol was incubated with rat liver microsomes in the presence of an NADPH-generating system and molecular oxygen, and the hydroxylated metabolites formed were analyzed by high-performance liquid chromatography (Fig. 1). A major metabolite was identified as 1′-hydroxybufuralol by comparing it with an authentic standard, and a minor metabolite (termed M-1) was formed through metabolism with liver microsomes of BNF-treated rats (Fig. 1B). Although attempts have not been made to identify the structure of M-1 in this study, the M-1 formation was suggested to be catalyzed by rat P450 enzymes based on the results of the effects of several chemical P450 inhibitors and anti-P450 antibodies (*vide infra*). As will be discussed below in detail, P450 1A1 had very high activity for the formation of M-1 in a reconstituted monooxygenase system (Fig. 1C).

The formation of 1′-hydroxybufuralol and M-1 was determined in liver microsomes of untreated male and female rats and male rats treated with PB, BNF, ISF, or DEX (Table 1). The activities of bufuralol 1′-hydroxylation in male rat liver microsomes were not significantly different from those in female rats. This metabolite formation was not increased significantly by several P450 inducers in male rats; rather, the activities were somewhat decreased in rats treated with PB and DEX, on the basis of P450 contents. In contrast, the formation of M-1 by rat liver microsomes was induced significantly by BNF, and moderately by ISF. These results suggested that M-1 formation is catalyzed mainly by

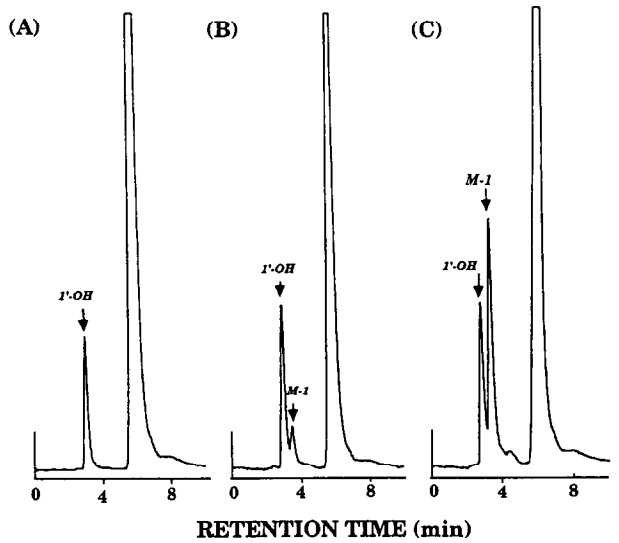

Fig. 1. High-performance liquid chromatographic separation of bufuralol and its metabolite in liver microsomes of untreated male rats (A), BNF-treated rats (B), and in a reconstituted monooxygenase system containing P450 1A1 (C). Liver microsomes (10 pmol equivalent of P450) and purified P450 1A1 (10 pmol of P450) in the reconstituted system were incubated with 0.4 mM bufuralol in the presence of an NADPH-generating system, and the metabolites formed were analyzed by high-performance liquid chromatography as described in Materials and Methods.

Table 1. Bufuralol 1'-hydroxylation and formation of M-1 by rat liver microsomes

Liver microsomes	Bufuralol hydroxylation (nmol product/min/nmol P450)	
	1'-Hydroxylation	M-1 Formation
Female rats		
Untreated	1.07 ± 0.13	0.015 ± 0.005
Male rats		
Untreated	1.00 ± 0.13	0.028 ± 0.007
PB-treated	0.59 ± 0.10*	0.017 ± 0.005
BNF-treated	1.14 ± 0.13	0.257 ± 0.038†
ISF-treated	1.23 ± 0.19	0.057 ± 0.006†
DEX-treated	0.52 ± 0.11†	0.024 ± 0.006

Bufuralol hydroxylation activities were determined, as described in Materials and Methods, with a substrate concentration of 0.4 mM. Each value represents the mean ± SD for three rats.

*,† Significantly different from untreated male rats: * $P < 0.05$, and $^{+}P < 0.01$.

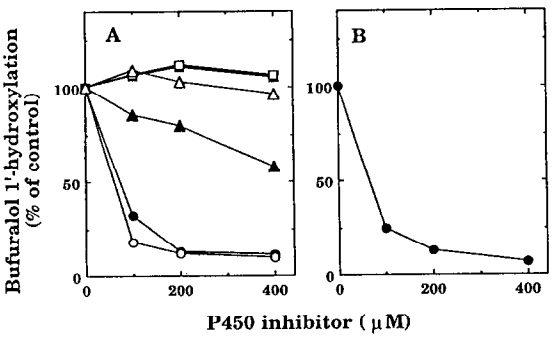

Fig. 2. (A) Effects of several chemical P450 inhibitors on bufuralol 1'-hydroxylation catalyzed by liver microsomes of untreated male rats. The inhibitors used were: SKF-525A (○), ANF (△), quinine (●), quinidine (▲), metyrapone (■), and methylpyrazole (□). (B) Effect of quinine on bufuralol 1'-hydroxylation catalyzed by liver microsomes of untreated female rats. Uninhibited activities in the absence of inhibitors were 1.24 nmol/min/nmol P450 for male rats and 1.02 nmol/min/nmol P450 for female rats. Each point represents the mean of duplicate determinations.

P450 1A1/2 in rat liver microsomes, while constitutive forms of P450 may play a role in bufuralol 1'-hydroxylation.

Effects of chemical inhibitors and anti-P450 antibodies on bufuralol hydroxylation activities in rat liver microsomes. Several P450 inhibitors, including SKF-525A (a nonspecific P450 inhibitor) [28], ANF (a selective inhibitor for P450 1A1/2) [28, 29], metyrapone (an inhibitor of P450 2B-type reactions) [28], methylpyrazole (a P450 2E1 inhibitor) [30], and quinine and quinidine (two P450 2D-dependent inhibitors) [31], were tested for their abilities to inhibit bufuralol hydroxylation activities catalyzed by liver microsomes of untreated male rats (Fig. 2). Only the effects of quinine were examined in female rats. Recent studies in the rat have suggested that quinine is a potent inhibitor for P450 2D1, while quinidine is not as potent [31]. The reverse is true for human P450 2D6 where quinidine has been

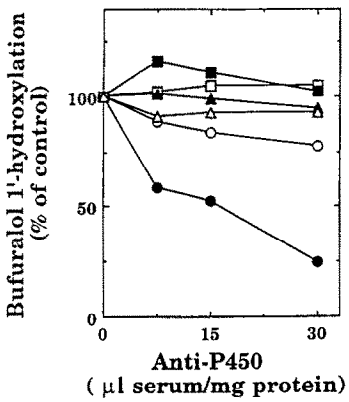

Fig. 3. Effects of anti-P450 antibodies on bufuralol 1'-hydroxylation activities catalyzed by liver microsomes of untreated male rats. Antibodies (antisera) used were: preimmune sera (□), anti-P450 2C11 (■), anti-P450 2D1 (●), anti-P450 3A2 (▲), anti-P450 1A1 (○), and anti-P450 1A2 (△). Uninhibited activity in the absence of antibodies was 1.15 nmol/min/nmol P450. Each point represents the mean of duplicate determinations.

shown to be a selective inhibitor for the reaction [31].

Quinine as well as SKF-525A, inhibited markedly the bufuralol 1'-hydroxylation activities in liver microsomes of untreated male rats (Fig. 2A). Quinidine, a known specific inhibitor of human P450 2D6, inhibited the activities very slightly. ANF, metyrapone and methylpyrazole did not inhibit the bufuralol 1'-hydroxylation catalyzed by liver microsomes of untreated male rats. A similar tendency for the inhibition of bufuralol 1'-hydroxylation by quinine was observed in liver microsomes of female rats (Fig. 2B). We also determined the effects of gestodene, a suicide inhibitor of P450 3A enzymes, on the bufuralol hydroxylation activities of rat liver microsomes and found that this chemical did not inhibit the activities after metabolic activation (data not shown) [32].

Several antibodies raised against rat P450 enzymes were examined for inhibition of bufuralol 1'-hydroxylation catalyzed by liver microsomes of male rats (Fig. 3). Anti-P450 2D1 antibodies inhibited very significantly the bufuralol 1'-hydroxylation catalyzed by liver microsomes of untreated male rats. Antibodies raised against other rat P450 enzymes, including P450 2C11, 3A2, and 1A2, did not inhibit the reaction, while anti-P450 1A1 inhibited the activities slightly. When male liver microsomes were replaced by those from untreated female rats, only anti-P450 2D1 inhibited the bufuralol 1'-hydroxylation reaction (data not shown).

As mentioned above, the formation of M-1 catalyzed by liver microsomes was induced very significantly in rats treated with BNF. To examine the possible roles of P450 1A enzymes in the reaction, we examined the effects of anti-P450 1A1 antibodies on the formation of M-1 as well as 1'-hydroxybufuralol by BNF-induced rat liver microsomes (Fig. 4). Since anti-P450 1A1 antibodies cross-reacted weakly with P450 1A2 and the bufuralol hydroxylation activities were catalyzed more by P450 1A1 than by P450 1A2 in the reconstituted system (*vide infra*), we used only anti-P450 1A1 antibodies for immunoinhibition experiments. Bufuralol 1'-hydroxylation was decreased to about 50% and the formation of M-1 was inhibited almost completely by anti-P450 1A1 antibodies in liver microsomes of BNF-treated rats (Fig. 4, A and B). Inclusion of ANF in the reaction mixture produced almost the same results (Fig. 4C).

Reconstitution of bufuralol hydroxylation by nine forms of rat P450 enzymes. The formation of 1'-hydroxybufuralol and M-1 was examined for reconstitution in systems containing nine forms of purified rat P450 enzymes (Table 2). In contrast to the results with liver microsomal experiments where

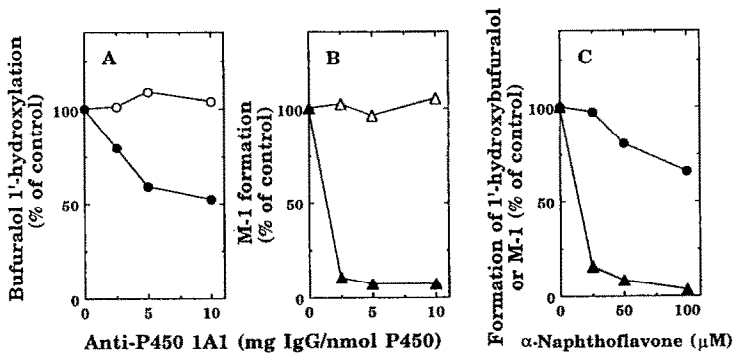

Fig. 4. Effects of anti-P450 1A1 antibodies (A and B) and ANF (C) on bufuralol hydroxylation activities catalyzed by liver microsomes of BNF-treated rats. Uninhibited activity in the absence of antibodies or ANF was 1.12 nmol/min/nmol P450 for bufuralol 1'-hydroxylation, and 0.285 nmol/min/nmol P450 for M-1 formation. Symbols: (A) effects of preimmune (○) and anti-P450 1A1 (●) antibodies on bufuralol 1'-hydroxylation; (B) effects of preimmune (△) and anti-P450 1A1 (▲) antibodies on the formation of M-1; and (C) effects of ANF on bufuralol 1'-hydroxylation (●) and M-1 formation (▲). Each point represents the mean of duplicate determinations.

Table 2. Bufuralol 1′-hydroxylation and formation of M-1 by a reconstituted monooxygenase system containing rat P450 enzymes

P450	Bufuralol hydroxylation (nmol product/min/nmol P450)	
	1′-Hydroxylation	M-1 Formation
P450 1A1	2.72 ± 0.08	3.080 ± 0.120
P450 1A2	0.47 ± 0.08	0.447 ± 0.056
P450 2B1	0.08 ± 0.08	ND*
P450 2B2	0.23 ± 0.09	ND
P450 2C6	0.31 ± 0.09	ND
P450 2C11	3.59 ± 0.58	ND
P450 2C12	ND	ND
P450 2D1	1.33 ± 0.32	ND
P450 3A2	0.18 ± 0.09	ND

Bufuralol hydroxylation activities were determined, as described in Materials and Methods, with a substrate concentration of 0.4 mM. Each value represents the mean ± range of duplicate determinations.

* Not detectable (the limits of detection for the formation of 1′-hydroxybufuralol and M-1 were 0.01 and 0.001 nmol/min/nmol P450, respectively).

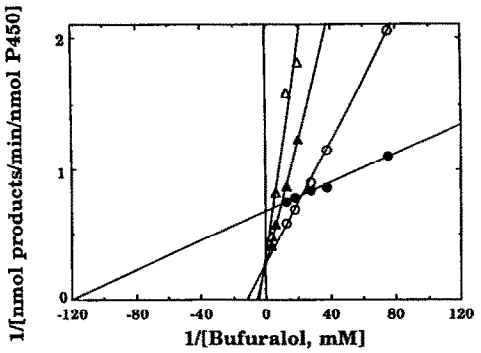

Fig. 5. Kinetic analysis of the hydroxylation of bufuralol by a reconstituted monooxygenase system containing P450 1A1 [(△) bufuralol 1′-hydroxylation, and (▲) M-1 formation], P450 2C11 [(○) bufuralol 1′-hydroxylation], and P450 2D1 [(●) bufuralol 1′-hydroxylation].

P450 2D1 appeared to be a principal enzyme in the bufuralol 1′-hydroxylation reaction, P450 2C11 gave the highest activities followed by P450 1A1 and P450 2D1 in a reconstituted monooxygenase system. The formation of M-1, on the other hand, was catalyzed largely by P450 1A1 in a reconstituted system; P450 1A2 also catalyzed M-1 formation, but not as much as P450 1A1.

Kinetic analysis for bufuralol hydroxylation by rat liver microsomal P450 enzymes. These results suggested that bufuralol 1′-hydroxylation is catalyzed mainly by P450 2D1, while P450 1A1 plays a major role in the formation of M-1 metabolite by rat liver microsomes. However, a question arises as to why P450 2C11 has the highest activities for bufuralol 1′-hydroxylation in the reconstituted system, although anti-P450 2C11 did not inhibit the activities catalyzed by liver microsomes of untreated male and female rats. The kinetic analysis for 1′-hydroxylation of bufuralol was determined in a reconstituted monooxygenase system containing P450 2D1, 2C11, and 1A1 (Fig. 5). In the case of P450 1A1, the formation of M-1 was also determined kinetically. Although the V_{max} values were higher for bufuralol 1′-hydroxylation in systems containing P450 2C11 and P450 1A1 than P450 2D1, the K_m value was the lowest in a system containing P450 2D1 (8.4 μM), as compared with systems containing P450 2C11 (83 μM) and P450 1A1 (230 μM) (Table 3). The K_m value for the formation of M-1 by a system containing P450 1A1 was determined to be 190 μM; the value was very similar to that for bufuralol 1′-hydroxylation.

In conclusion, the present work showed that P450 enzymes have different roles in the hydroxylation of bufuralol in rat liver microsomes. As reported previously in several laboratories [5, 9], P450 2D1 was confirmed to be a major enzyme involved in the bufuralol 1′-hydroxylation in liver microsomes of untreated male and female rats. P450 1A1 was determined to be a principal enzyme in the formation of M-1 (the structure of this metabolite is not known at present) and P450 1A2 was also found to be involved in M-1 formation although not as much as P450 1A1. In a reconstituted monooxygenase system containing nine forms of rat P450 enzymes, P450 2C11 was found to have the highest activities for bufuralol 1′-hydroxylation, although anti-P450 2C11 did not inhibit the activities catalyzed by rat liver

Table 3. Kinetic analysis of bufuralol 1′-hydroxylation and formation of M-1 by a reconstituted monooxygenase system containing P450 1A1, 2D1, and 2C11

	Bufuralol hydroxylation			
	1′-Hydroxybufuralol		M-1 Formation	
	K_m (mM)	V_{max} (nmol/min/nmol P450)	K_m (mM)	V_{max} (nmol/min/nmol P450)
P450 2D1	0.008	1.49		
P450 2C11	0.083	3.70		
P450 1A1	0.230	3.09	0.190	3.80

microsomes, as was the case for debrisoquine 4-hydroxylase [10]. Kinetic analysis, however, indicated that the K_m value of P450 2D1 for bufuralol 1′-hydroxylation was lower than those of P450 2C11 and 1A1, and thus the major role of P450 2D1 in the reaction was confirmed. It is interesting to note that a female-specific form of P450 2C12 did not catalyze bufuralol hydroxylation, although there were no sex-related differences in the reaction. These results suggested again that P450 2D1, but not P450 2C11, is a principal enzyme in bufuralol 1′-hydroxylation in rat liver microsomes. The potent inhibition by anti-P450 2D1 antibodies of bufuralol 1′-hydroxylation catalyzed by liver microsomes supported the above view. Therefore, the present results suggest that different P450 enzymes are involved in the hydroxylation of bufuralol in rat liver microsomes; kinetic analysis, as well as immunoinhibition and chemical inhibition experiments, may be of great importance for determining the major contribution of P450 enzymes in drug hydroxylation reactions.

Acknowledgement—Part of this work was supported by grants from the Ministry of Education, Science, and Culture of Japan.

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