

Metabolism of a New Local Anesthetic, Ropivacaine, by Human Hepatic Cytochrome P450

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Background: Ropivacaine is a local anesthetic with a long duration of action. Although it is less toxic than bupivacaine, local anesthetic toxicity is possible when the plasma concentration is increased. Because ropivacaine is an amide-type local anesthetic, it is metabolized by cytochrome P450 (P450) in the liver, and its elimination and plasma concentration can be dependent on the level of P450. The purpose of this investigation was to elucidate the metabolism of ropivacaine by human hepatic P450.

Methods: The metabolism of ropivacaine was compared using recombinant human and purified rat hepatic P450 isozymes. An inhibition study using antibodies against rat P450 was performed using hepatic microsomes from human and rat to identify which P450s are involved in ropivacaine metabolism.

Results: Ropivacaine was metabolized to 2',6'-pipecoloxylidide (PPX), 3'-hydroxyropivacaine (3'-OH Rop), and 4'-hydroxyropivacaine (4'-OH Rop) by hepatic microsomes from human and rat. PPX was a major metabolite of both human and rat hepatic microsomes. In a reconstituted system with rat P450, PPX was produced by CYP2C11 and 3A2, 4'-OH Rop by CYP1A2, and 3'-OH Rop by CYP1A2 and 2D1. Formation of PPX in rat hepatic microsomes was inhibited by anti CYP3A2, but not by CYP2C11 antibody, and formation of 3'-OH Rop was inhibited by CYP1A2 and 2D1 antibodies. Anti CYP3A2 and 1A2 antibodies inhibited the formation of PPX and 3'-OH Rop in human hepatic microsomes, respectively. Recombinant hu-

man P450s expressed in lymphoblast cells were used for further study. CYP3A4 and 1A2 formed the most PPX and 3'-OH Rop, respectively. Ropivacaine N-dealkylation and 3'-hydroxylation activities correlated well with the level of CYP3A4 and 1A2 in human hepatic microsomes, respectively.

Conclusions: Ropivacaine was metabolized to PPX, 3'-OH Rop, and 4'-OH Rop by hepatic P450. PPX was a major metabolite in human hepatic microsomes. CYP3A4 was involved in producing PPX. CYP1A2 was involved in the formation of 3'-OH Rop in human hepatic microsomes. (Key words: Anesthetics, local: ropivacaine. Biotransformation: ropivacaine. Liver: microsomes. Metabolism: CYP1A2; cytochrome P450; CYP3A4; genetic factors; immunoinhibition.)

ROPIVACAINE is an amide-type local anesthetic with a structure similar to that of mepivacaine and bupivacaine.¹ Although ropivacaine is less toxic than bupivacaine,² it produces a profile of local anesthetic toxicity similar to that of other local anesthetics, including convulsions and hypotension.³ When ropivacaine is administered to humans, there is a considerable interindividual variability in the plasma concentration, resulting in marked differences in the incidence of side effects.²

Because amide-type local anesthetics are metabolized predominantly by the microsomal cytochrome P450 (P450) in the liver, ropivacaine also could be metabolized by P450. Additionally, human hepatic microsomes contain multiple forms of P450, and the involvement of genetic polymorphisms of oxidation as the cause of interindividual variations of elimination of exogenous compounds has been reported.⁴ Usually, ropivacaine is administered clinically as a regional anesthetic with other agents, including general anesthetics and cardiovascular agents.^{5,6} Sometimes, one drug inhibits the metabolism of other drugs when they are metabolized by the same P450.⁷ Some of these agents influence P450 activity, which may in turn affect the plasma concentration of ropivacaine,⁷⁻¹⁰ inducing central nervous system and/or cardiac side effects. Therefore, it is clinically important to know which P450 isoform metabolizes individual drugs. The cur-

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rent study was undertaken to define the enzymes in human liver that are responsible for metabolism of ropivacaine by using several forms of human hepatic P450 isozymes synthesized from recombinant DNA and to compare the enzyme specificities with those of rat hepatic P450.

Materials and Methods

Chemicals

The experimental protocol was approved by the Institutional Ethical Committee. Ropivacaine: (S)-enantiomer of 1-propyl-2',6'-pipecoloxylidide, and its metabolites, 2',6'-pipecoloxylidide (PPX), 3'-hydroxyropivacaine (3'-OH Rop), and 4'-hydroxyropivacaine (4'-OH Rop) were gifts from the Fujisawa Pharmaceuticals Co., Ltd (Osaka, Japan). Recombinant human P450s expressed in human lymphoblast cells were obtained from Gentest (Woburn, MA). These were supplied as microsomes. Dilauroylphosphatidylcholine and dioleoylphosphatidylcholine were obtained from Sigma Chemical Co. (St. Louis, Mo). Phosphatidylserine (bovine) was obtained from PL Biochemicals (Milwaukee, WI). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from the Oriental Yeast Co. (Tokyo, Japan). A reverse-phase octadecasil column (TSK gel ODS-120T, 4.6 × 250 mm) was obtained from the Tosoh Corp. (Tokyo, Japan). Other reagents and organic solvents were obtained from the Wako Pure Chemical Industries (Tokyo, Japan).

Preparation of Microsomes and Purification of P450 from Rat Hepatic Microsomes

Human hepatic microsomes were prepared from 15 liver samples as described for the preparation of rat hepatic microsomes.¹¹ These liver samples were obtained from cancer patients undergoing liver resection. Tissues were selected from areas of the liver that were visually free of tumor, frozen within 60 min, and stored at -80°C until used. Samples that had a specific level of P450 above 0.1 nmol/mg of protein were used in this study. Rat hepatic microsomes were prepared, and rat P450s were purified as described.¹¹⁻¹³ NADPH-P450 reductase and cytochrome b₅ were purified as described.¹⁴

Assay of Ropivacaine Metabolism

The ropivacaine monooxygenase activity was measured as we reported for lidocaine.¹⁵ Ropivacaine (0.1 mM) was incubated with hepatic microsomes (200 µg of protein) from rats or humans. Metabolic activities of hepatic microsomes are proportional to 1.0 mg of microsomal protein. Purified rat hepatic P450 (30 pmol) and NADPH-P450 reductase (0.3 units) were reconstituted with dilauroylphosphatidylcholine (10 µg). The catalytic activity of each P450 is proportional to the hemoprotein concentration (0–60 pmol/0.5 ml). Microsomes from lymphoblast cells (500 µg of protein) were used to recombinant human P450 assay. CYP3A2** has low activity in the conventional reconstituted system described above,¹⁷ so we used a modified reconstituted system containing a mixture of phospholipids (10 µg) consisting of dilauroylphosphatidylcholine, dioleoylphosphatidylcholine, and phosphatidylserine (1:1:1) and sodium cholate (100 µg). Microsomes and reconstituted P450 were reacted with ropivacaine (0.1 mM) in the presence of NADPH (0.4 mM) at 37°C for 60 and 15 min, respectively. The formation of ropivacaine metabolites was linear up to 90 min when rat and human hepatic microsomes were used. It was also linear up to at least 15 min when reconstituted P450 was used. The final volume of the reaction mixture was 0.5 ml in 0.1 M potassium phosphate (pH 7.4). The reaction was stopped by adding 1 N NaOH (50 µl). The metabolites were extracted with ethyl acetate (2 ml). The organic phase was evaporated *in vacuo*, and the residue was dissolved in elution buffer for high-performance liquid chromatography (200 µl). One hundred microliters of the solution was injected onto an high-performance liquid chromatography apparatus with a ODS-120T column and isocratically eluted with 0.1 M potassium phosphate buffer (pH 3.0) and acetonitrile (9:1, v/v). Chromatography was done at a flow rate of 1.5 ml/min at 60°C, and the metabolites were monitored at 214 nm.

In our assay, internal standard was not used for measuring ropivacaine metabolic activity. The amount of ropivacaine metabolites was calculated by comparison of their peak area as calculated by a data processor with those of authentic ropivacaine metabolites such as PPX, 3'-OH Rop, and 4'-OH Rop. The same analytical method was used in our previous reports for measuring metabolites of lidocaine and aminopyrine.^{14,15} Standard curve for PPX was linear between 0–5 µM, standard curves for 3'-OH Rop and 4'-OH Rop were also linear between 0 and 2 µM. Coefficients of correlation of these

** The nomenclature used here for the P450 enzymes is that described by Nelson *et al.*¹⁶

standard curves were more than 0.999, and limit of quantification was 0.01 nanomole of products per minute per nanomole of P450.

Other Methods

Antibodies against rat CYP1A2, 2B1, 2C11, 2D1, and 3A2 were raised in a rabbit and immunoglobulin G (IgG) as reported.¹⁸ P450 was measured by immunoblotting as described previously.¹⁸ Antibodies (20, 40, 60, 80, and 100 μ g of IgG) were incubated with the microsomes (200 μ g of protein) for 20 min at room temperature.

Analysis

Rates of metabolism are expressed as nanomoles of product formed per minute per milligram of protein, or per nanomole of P450. The relationships between ropivacaine metabolic activity and the content of P450 enzymes were examined by the linear least-squares correlation analysis.

Results

Separation of Ropivacaine Metabolites by High-performance Liquid Chromatography

The high-performance liquid chromatography profile of the ropivacaine metabolites produced by human hepatic microsomes is shown in figure 1A. Peaks of ropivacaine metabolites were identified by comparison with those of authentic PPX, 3'-OH Rop, and 4'-OH Rop. The peak areas were increased in proportion to the amounts of hepatic microsomes present. These metabolites did not appear when ropivacaine was incubated with human hepatic microsomes without NADPH (fig. 1B), suggesting that those metabolites were produced by NADPH-dependent P450 monooxygenase (fig. 2). These ropivacaine metabolites also were formed when ropivacaine was intravenously administered into humans.^{††} Formation rates of PPX, 4'-OH Rop, and 3'-OH Rop by human hepatic microsomes from one liver sample were 0.560, 0.030, and 0.047 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹, respectively.

Ropivacaine Metabolic Activity of Purified Rat Hepatic P450

The ropivacaine metabolic activity of purified rat hepatic P450s is shown in table 1. CYP2C11 is male-

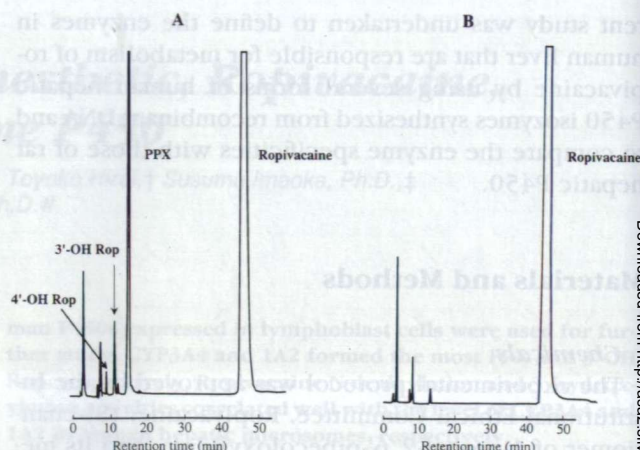


Fig. 1. Elution profiles of ropivacaine and its metabolites by high-performance liquid chromatography. (A) Chromatogram of ropivacaine metabolites generated by human hepatic microsomes. Ropivacaine (0.1 mM) was incubated with human hepatic microsomes (200 μ g of protein) and reduced nicotinamide adenine nucleotide phosphate (0.4 mM) at 37°C for 60 min. (B) Chromatogram of the blank assay. The incubation mixtures were the same as in A except for the omission of reduced nicotinamide adenine nucleotide phosphate.

specific,¹⁹ and it formed high levels of PPX. The formation of PPX by CYP3A2, another major constitutive P450 isozyme in microsomes from male rats,¹¹ was also high in the modified reconstituted system. The ropivacaine N-dealkylation activity of CYP1A2, 2A2, 2B1, and 2D1 was low and undetectable using CYP2B2 nor 2E1.

CYP1A2 had low activity toward ropivacaine 4'-hydroxylation, but the other P450 isozymes did not. CYP1A2 and 2D1 had high activity toward ropivacaine 3'-hydroxylation. CYP2B1, 2C11, and 2E1 had slight ropivacaine 3'-hydroxylation activity.

The Effect of P450 Antibody on the Ropivacaine Metabolic Activity of Rat Hepatic Microsomes

We performed an immunoinhibition study using antibodies against rat CYP1A2, 2C11, 2D1, and 3A2, because these P450 isozymes had high ropivacaine metabolic activities. Ropivacaine N-dealkylation was more than 80% inhibited by an antibody against CYP3A2 (fig. 3). However, this activity was not inhibited by an antibody against CYP2C11, although CYP2C11 had high ropivacaine N-dealkylation activity (table 1). We showed that CYP2C11 had high lidocaine N-dealkylation activity, whereas its antibody did not inhibit it,²⁰ although anti CYP2C11 antibody can inhibit

^{††} Fujisawa Pharmaceuticals Co. Ltd.: Personal communication. 1993.

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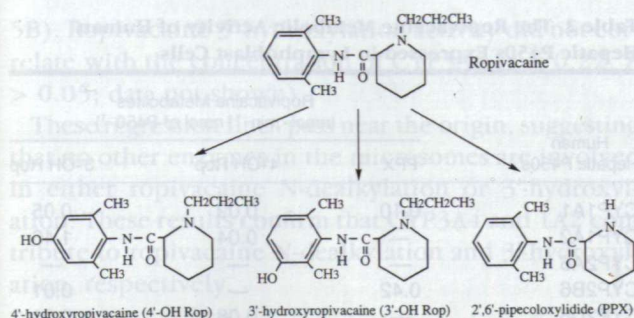


Fig. 2. Metabolic pathways of ropivacaine by human hepatic cytochrome P450.

testosterone 2 α - and 16 α -hydroxylation activities, which are catalyzed by CYP2C11 in microsomes from untreated rat liver.¹⁸ These results suggested that CYP2C11 was not involved in ropivacaine N-dealkylation in rat hepatic microsomes. Ropivacaine 4'-hydroxylation was about 70% inhibited by anti CYP3A2 antibody. It was also 40% inhibited by antibodies against CYP1A2 and 2D1 (fig. 3), although neither purified CYP2D1 nor 3A2 had ropivacaine 4'-hydroxylation activity. CYP2D1 and 3A2, as well as CYP1A2, may be involved in ropivacaine 4'-hydroxylation in rat hepatic microsomes. The ropivacaine 4'-hydroxylation activity of rat hepatic microsomes was very low, and those of CYP2D1 and 3A2 could be undetectable under our reaction conditions (table 1). Ropivacaine 3'-hydroxylation was almost completely inhibited by antibodies against CYP2D1 as well as CYP1A2 (fig. 3), suggesting that both of those P450 isozymes are involved in ropivacaine 3'-hydroxylation. These results are consistent with those obtained using purified rat hepatic P450 (table 1).

The Effect of P450 Antibody on the Ropivacaine Metabolic Activity of Human Hepatic Microsomes

Antibodies against rat hepatic P450s were useful for detecting P450 isozymes involved in ropivacaine metabolism. We used these P450 antibodies to identify the P450 isozymes involved in ropivacaine metabolism in human hepatic microsomes. Antibody against CYP3A2 inhibited both ropivacaine N-dealkylation and the 4'-hydroxylation activity of human hepatic microsomes by more than 80% (fig. 4). We showed that antibody against CYP3A2 specifically reacts with CYP3A4 in human hepatic microsomes and that CYP3A2 and 3A4 are immunochemically related.¹⁵ Antibodies

Table 1. The Ropivacaine Metabolic Activity of Purified Rat Hepatic P450s

Purified Rat Hepatic P450s	Ropivacaine Metabolites (nmol · min ⁻¹ · nmol of P450 ⁻¹)		
	PPX	4'-OH Rop	3'-OH Rop
CYP1A2	0.089	0.044	0.341
CYP2A2	0.086	—	—
CYP2B1	0.044	—	0.030
CYP2B2	—	—	—
CYP2C11	0.400	—	0.021
CYP2D1	0.038	—	0.140
CYP2E1	—	—	0.039
CYP3A2	0.041	—	—
CYP3A2*	0.310	—	—

Values are expressed as the mean of the two experiments.

— = values < 0.01 nanomole per minute per nanomole of cytochrome P450; PPX = 2',6'-pipecoloxylidide; 4'-OH Rop = 4'-hydroxyropivacaine; 3'-OH Rop = 3'-hydroxyropivacaine.

The reaction mixture containing rat hepatic cytochrome P450 (30 pmol), NADPH-cytochrome P450 reductase (0.3 units), and dilauroylphosphatidylcholine (10 μ g) was incubated for 15 min at 37°C in 0.1 M potassium phosphate buffer, pH 7.4. Ropivacaine metabolites were extracted with ethyl acetate and analyzed by HPLC.

* Mixture of phospholipids: dilauroylphosphatidylcholine, dioleoylphosphatidylcholine, and phosphatidylserine (1:1:1) were used instead of dilauroylphosphatidylcholine.

against CYP1A2, 2B1, 2C11, and 2D1 inhibited neither ropivacaine N-dealkylation nor 4'-hydroxylation. These results suggest that ropivacaine is selectively N-dealkylated and hydroxylated at position 4 by CYP3A4 in human hepatic microsomes. Antibody against CYP1A2

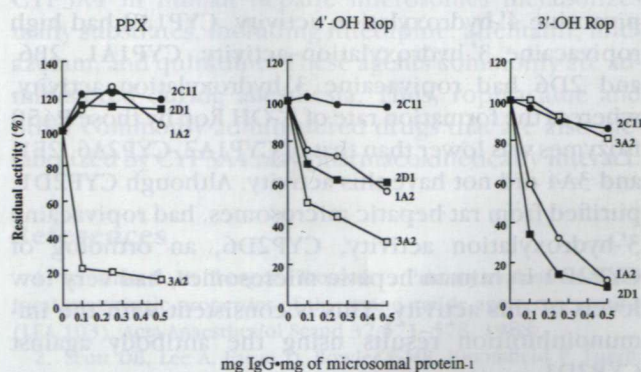


Fig. 3. Effect of antibodies against CYP1A2, 2C11, 2D1, and 3A2 on the catalytic activities of rat hepatic microsomes. The concentration of ropivacaine was 0.1 mM. The turnover rates of PPX, 4'-hydroxyropivacaine, and 3'-hydroxyropivacaine formed by rat hepatic microsomes without antibodies were 3.60, 0.038, and 0.400 nanomoles per minute per milligram of microsomal protein, respectively.

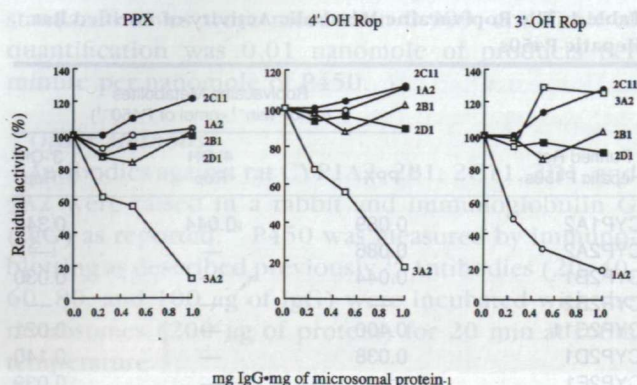


Fig. 4. Effects of antibodies against CYP1A2, 2B1, 2C11, 2D1, and 3A2 on the catalytic activities of human hepatic microsomes. The ropivacaine concentration was 0.1 mM. The turnover rates of PPX, 4'-hydroxyropivacaine, and 3'-hydroxyropivacaine formation by human hepatic microsomes without antibodies were 0.560, 0.030, and 0.047 nanomoles per minute per milligram of microsomal protein, respectively.

inhibited ropivacaine 3'-hydroxylation by more than 80%. Antibodies against CYP2B1, 2C11, 2D1, and 3A2 did not inhibit this reaction (fig. 4), suggesting that ropivacaine 3'-hydroxylation is catalyzed selectively by CYP1A2.

Ropivacaine Metabolic Activity of Human Hepatic P450

The ropivacaine metabolic activity of seven different human hepatic P450s expressed in human lymphoblasts is shown in table 2. CYP3A4 catalyzed ropivacaine N-dealkylation, and CYP1A1 and 2B6 had much less activity. CYP1A1, 1A2, 2D6, and 3A4 had low ropivacaine 4'-hydroxylation activity. CYP1A2 had high ropivacaine 3'-hydroxylation activity. CYP1A1, 2B6, and 2D6 had ropivacaine 3'-hydroxylation activity, whereas the formation rate of 3'-OH Rop by those P450 isozymes was lower than that of CYP1A2. CYP2A6, 2E1, and 3A4 did not have this activity. Although CYP2D1, purified from rat hepatic microsomes, had ropivacaine 3'-hydroxylation activity, CYP2D6, an ortholog of CYP2D1 in human hepatic microsomes, had very low levels of this activity. This is consistent with the immunoinhibition results using the antibody against CYP2D1.

Correlation of Ropivacaine Metabolic Activity with the Level of CYP3A4 and 1A2

The catalytic and inhibition studies described above suggested that ropivacaine is selectively N-dealkylated

Table 2. The Ropivacaine Metabolic Activity of Human Hepatic P450s Expressed in Lymphoblast Cells

Human Hepatic P450s	Ropivacaine Metabolites (nmol · min ⁻¹ · nmol of P450 ⁻¹)		
	PPX	4'-OH Rop	3'-OH Rop
CYP1A1	0.10	0.09	0.05
CYP1A2	—	0.04	1.46
CYP2A6	—	—	—
CYP2B6	0.42	—	0.01
CYP2D6	—	0.08	0.01
CYP2E1	—	—	—
CYP3A4	3.20	0.09	—

Values are expressed as the mean of the two experiments. The reaction mixture was the same as that described in the footnotes to table 1, except that recombinant human P450s expressed in human lymphoblast cells were used. They were supplied as microsomes, containing 500 µg of protein.

— = values <0.01 nanomole per minute per nanomole of cytochrome P450. The abbreviations for the ropivacaine metabolites are as expressed in table 1.

by human CYP3A4, and hydroxylated at position 3 by human CYP1A2. To confirm this, the levels of ropivacaine N-dealkylation and 3'-hydroxylation activities in individual human hepatic microsomes were compared with the immunochemically estimated levels of CYP3A4 and 1A2, respectively. The concentration of CYP3A4 was determined using an antibody against rat CYP3A2 as described.¹⁵ There were significant linear correlations between ropivacaine N-dealkylation activity and the CYP3A4 content ($r = 0.88$, $P < 0.0001$; fig. 5A) as well as between ropivacaine 3'-hydroxylation activity and CYP1A2 level ($r = 0.88$, $P < 0.0001$; fig.

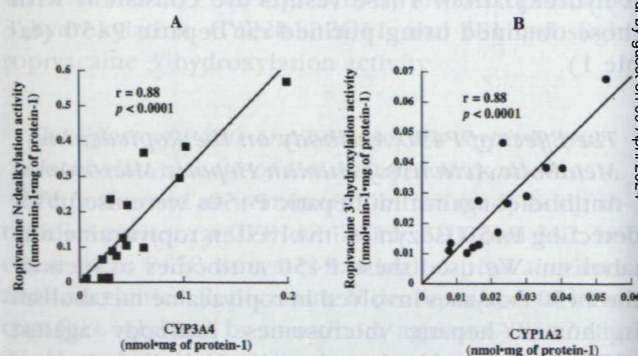


Fig. 5. (A) Correlation of ropivacaine N-dealkylation with the level of CYP3A4. The CYP3A4 level was determined by Western blotting analysis with antibody against CYP3A2. The linear regression correlation coefficient was 0.88 ($P < 0.0001$). (B) Correlation of ropivacaine 3'-hydroxylation with the level of CYP1A2. The linear regression correlation coefficient was 0.88 ($P < 0.0001$).

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5B). Ropivacaine 3'-hydroxylation activity did not correlate with the concentration of CYP3A4 ($r = 0.22$, $P > 0.05$; data not shown).

These regression lines pass near the origin, suggesting that no other enzymes in the microsomes are involved in either ropivacaine N-dealkylation or 3'-hydroxylation. These results confirm that CYP3A4 and 1A2 contribute to ropivacaine N-dealkylation and 3'-hydroxylation, respectively.

Discussion

In this study, we found that human hepatic P450 specifically metabolized ropivacaine. PPX was the major metabolite commonly found in humans and rats, and it was formed predominantly by P450 in the same gene family, CYP3A4 and 3A2. CYP3A2 is ortholog to CYP3A4, and they are structurally and immunochemically related each other.²¹ Antibodies against CYP3A2 also react with CYP3A4 in human hepatic microsomes and can be used to determine the level of CYP3A4 in human hepatic microsomes.¹⁵

3'-OH Rop and 4'-OH Rop also were formed in microsomes from human and rat liver, and P450 isozymes were involved in these reactions. We have determined the enzyme specificity for lidocaine metabolism, and CYP3A4 is the major P450 isozyme involved in lidocaine N-dealkylation in human hepatic microsomes.¹⁵ CYP3A4 plays a major role in the oxidation of many drugs including nifedipine, alfentanil, midazolam, and quinidine.²¹⁻²⁴ These drugs may compete with ropivacaine for drug metabolism. In this study, ropivacaine also was metabolized by CYP3A4 in human hepatic microsomes to the N-dealkylated form, PPX. PPX has been detected in humans after mepivacaine and bupivacaine administration,²⁵ suggesting that the N-dealkylation of these anesthetics is catalyzed by CYP3A4. CYP3A4 is inducible by drugs such as barbiturate and dexamethasone.²¹ When this P450 is induced, metabolism of ropivacaine will be increased.

CYP2D6 is present in human hepatic microsomes and contributes to genetic polymorphisms in debrisoquine metabolizing activities.⁴ The ropivacaine 3'-hydroxylation activity of CYP2D6 was very low, and the formation of 3'-OH Rop was not inhibited by antibody against CYP2D1, suggesting that CYP2D6 is not involved in ropivacaine 3'-hydroxylation in human hepatic microsomes. These results contradict those obtained from rats, because CYP2D1 is involved in ropivacaine 3'-hydroxylation in rat hepatic microsomes.

We showed that CYP2D1 is involved in lidocaine 3'-hydroxylation in rat hepatic microsomes.²⁶ However, lidocaine 3'-hydroxylation activity in human hepatic microsomes constituted less than one-tenth of that in rat hepatic microsomes,^{15,20} and the plasma concentration of aromatic 3'-hydroxylated lidocaine in human is very low after the intravenous administration of lidocaine.²⁷ In this study, the formation rate of 3'-OH Rop was greater by rat than by human hepatic microsomes. Because 3'-hydroxylation activity of lidocaine and ropivacaine by human hepatic microsomes was low and plasma concentration of 3'-hydroxylated lidocaine is also low, we speculate that aromatic ring hydroxylation at position 3 of lidocaine and ropivacaine is not the major metabolic pathway in humans, and that CYP2D6 is not involved in the metabolism of these agents. CYP1A2 is involved in ropivacaine 3'-hydroxylation commonly in human and rat hepatic microsomes.

Although ropivacaine 4'-hydroxylation by human hepatic microsomes was approximately 70% inhibited by antibody against CYP3A2 (fig. 4), ropivacaine 4'-hydroxylation activity of CYP3A4 was as high as that of CYP1A1 and 2D6. These results suggest that CYP3A4 is responsible for a significant fraction of 4'-OH Rop, whereas other P450 isozymes also could be involved in this reaction.

In summary, we showed that ropivacaine is N-dealkylated and aromatic-ring hydroxylated at positions 3 and 4 to PPX, 3'-OH Rop, and 4'-OH Rop, respectively, in human hepatic microsomes and that PPX was the major metabolite. The specific P450 isozymes, CYP3A4 and 1A2, are involved in these metabolic pathways. CYP3A4 in human hepatic microsomes metabolizes many substrates, including nifedipine, alfentanil, midazolam, and quinidine. These agents commonly are administered during anesthesia. Thus, ropivacaine and other commonly administered drugs that are also metabolized by CYP3A4 may pharmacokinetically interact.

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