0090-9556/00/2804-0383–385$02.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 28, No. 4
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Short Communication

Oxidative Metabolism of Bupivacaine into Pipecolylxylidine in Humans Is Mainly Catalyzed by CYP3A

(Received June 22, 1999; accepted December 6, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:

Bupivacaine is used to provide prolonged anesthesia and postoperative analgesia. The human cytochrome P450 (CYP) involved in bupivacaine degradation into pipecolylxylidine (PPX), its major metabolite, has, to our knowledge, never been described. Microsome samples were prepared from six human livers and incubated in the presence of bupivacaine. The concentrations of PPX in the microsomal suspensions were assessed, and K_m and V_{max} values were calculated. Bupivacaine incubations were then performed with specific CYP substrates and inhibitors. For each sample of hepatic microsomes, the correlation between the rate of PPX formation and the corresponding erythromycin *N*-demethylase activity was analyzed. Finally, an immunoinhibition study using an anti-rabbit CYP3A6 antibody and assays with cDNA-expressed human CYP were conducted. The apparent K_m and V_{max} values of bupivacaine were, respectively, 125 μM and 4.78 nmol/min/mg of microsomal protein. The strongest inhibition of bupivacaine metabolism was obtained for troleandomycin (−95% at 50 μM), a specific CYP3A inhibitor. The correlation between PPX formation and erythromycin *N*-demethylase activity showed an *R* value of 0.99 whereas anti-rabbit CYP3A6 antibody inhibited the degradation of bupivacaine into PPX by 99%. Finally, CYP1A2 and CYP2E1 cDNA-expressed forms of human CYP did not allow PPX formation, CYP2C19 and CYP2D6 produced only small amounts whereas CYP3A4 most efficiently metabolized bupivacaine into PPX. These results demonstrated that bupivacaine degradation into PPX was mediated in humans by CYP3A.

Bupivacaine, introduced in 1963, is a widely used amide local anesthetic. Its long duration of action as well as its tendency to provide more sensory than motor block has made it a popular drug for providing prolonged local anesthesia (Catterall and Mackie, 1996).

Bupivacaine is *N*-dealkylated in humans to form a less toxic metabolite pipecolylxylidine (PPX)[1], which is hydroxylated and then forms glucuronide conjugates. Very little unchanged bupivacaine is eliminated in the urine (Reynolds, 1971; Boyes, 1975). In a previous study, we demonstrated in mice that Cyp3a is involved in the hepatic degradation of bupivacaine (Attolini et al., 1997). Lidocaine and ropivacaine, two structural analogs of bupivacaine, are both oxidized in humans by CYP3A (Bargetzi et al., 1989; Ekstrom and Gunnarsson, 1996). There is very little information on the biotransformation of bupivacaine and to our knowledge, the enzymes involved in the metabolism of bupivacaine in humans have not been identified.

The aim of this study was to identify, by means of correlation, inhibition, and immunoinhibition studies and cDNA-expressed human CYP, the CYP isoforms catalyzing bupivacaine oxidative metabolism into PPX in humans.

[1] Abbreviations used are: PPX, pipecolylxylidine; HL, human liver; CYP, cytochrome P450.

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Materials and Methods

Chemicals. Bupivacaine and PPX were kindly provided by Janssen (Boulogne, France). Caffeine, coumarin, tolbutamide, phenytoin, dextromethorphan, *p*-nitrophenol, troleandomycin, and NADPH (β-nicotinamide adenine dinucleotide phosphate) were obtained from Sigma Chemical Corporation (St. Quentin Fallavier, France). The other reagents were of the best commercially available quality.

Human Liver (HL) Microsomal Preparations. Specimens HL1, HL7, HL20, HL23, HL26, and HL30 were obtained, respecting strict ethical conditions, from organ donors when liver was not suitable for transplantation. Microsomal preparations were performed by differential ultracentrifugation (van der Hoeven and Coon, 1974). Microsomal protein concentrations were determined by the method of Bradford (Bio-Rad Protein Assay Kit, Bio-Rad, Ivry sur Seine, France) using serum albumin as standard (Bradford, 1976).

Bupivacaine Oxidative Metabolism. Hepatic microsomes from individual livers or a pool of six microsomal preparations were suspended in a final volume of 500 μl at a concentration of 1 mg of microsomal protein/ml in 0.1 M potassium phosphate buffer (pH 7.4) in the presence of bupivacaine. After 5 min at 37°C, the reaction was initiated with 1 mM NADPH and stopped after 15 min by addition of 250 μl of cold methanol. All experiments were performed in triplicate. The linearity of bupivacaine metabolism was tested before this study. After centrifugation, 600 μl of supernatant was analyzed as described previously (Lorec et al., 1994).

Determination of the K_m of Bupivacaine Degradation into PPX. The pool

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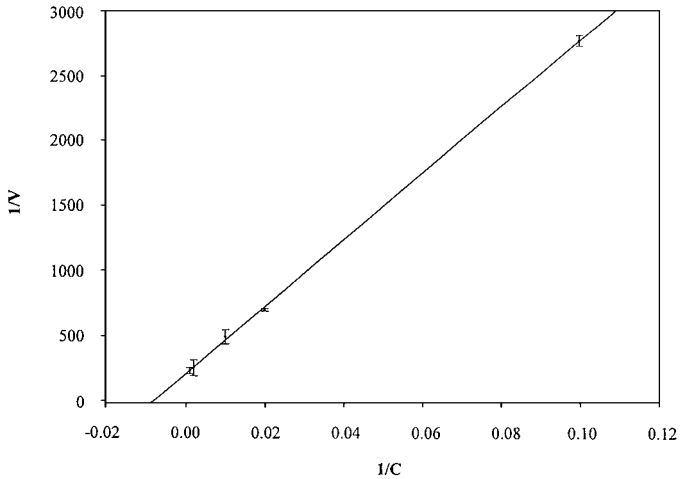

FIG. 1. *Kinetic constants of PPX formation from bupivacaine in HL microsomes, assessed using Lineweaver-Burk plots.*

The apparent K_m and V_{max} values for bupivacaine metabolism were, respectively, 125 μM and 4.78 nmol/min/mg of microsomal protein. C: concentration (μM). V: turnover (nanomole per minute per milligram of microsomal protein).

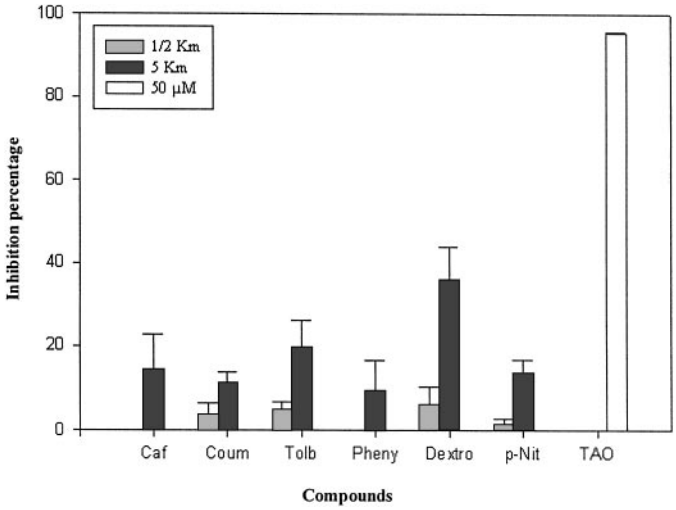

FIG. 2. *Inhibitory effect of various compounds on PPX formation.*

No inhibitory effect was observed for the low concentration of caffeine and phenytoin. Caf: caffeine. Coum: coumarin. Tolb: tolbutamine. Pheny: phenytoin. Dextro: dextromethorphan. *p*-Nit: *p*-nitrophenol. TAO**:** troleandomycin.

of six microsomal preparations was incubated, as described above, with various concentrations of bupivacaine (10–1000 μM). K_m and V_{max} values were calculated by means of Lineweaver-Burk representation. All experiments were performed in triplicate.

Inhibition of Bupivacaine Metabolism by Specific CYP Substrates. Bupivacaine (K_m concentration: 125 μM) was incubated, as described above, in presence of specific CYP substrates and inhibitors used at the concentrations of 1/2 or 5 times the bupivacaine K_m (caffeine, coumarin, tolbutamide, phenytoin, dextromethorphan, and *p*-nitrophenol) and microsomes from pooled HLs. To be specific for CYP3A, troleandomycin was used at 50 μM and preincubated for 15 min at 37°C with NADPH before the addition of bupivacaine. All experiments were performed in triplicate.

Correlation Analysis. The correlation between bupivacaine metabolism into PPX and erythromycin *N*-demethylase activity (Guengerich, 1987), representative of CYP3A activity, was studied using the K_m concentration of bupivacaine (125 μM) for the six HL microsome samples. All experiments were performed in triplicate. The linear regression coefficient (R) was used as correlation parameter.

Immunoinhibition Study. An immunoinhibition study was performed using an anti-rabbit CYP3A6 antibody (Zhou-Pan et al., 1993). Bupivacaine was incubated as described above in presence of various CYP3A6 antibody concentrations (0–5 mg/ml). The experiments were performed using a pool of the six HL microsomes. Preimmune IgG was used to keep the total amount of added IgG constant. All experiments were performed in triplicate.

Assays with cDNA-Expressed human CYP. Microsomes of insect cells overexpressing human CYP2C19, CYP2D6, and NADPH cytochrome P450 reductase were purchased from PanVera Corporation (Madison, WI) as well as human purified recombinant CYP1A2, CYP2E1, CYP3A4, NADPH cytochrome P450 reductase, and cytochrome b_5 reductase. The metabolism of bupivacaine, at K_m concentration (125 μM), was performed as described above and according to the supplier.

Results and Discussion

Apparent K_m and V_{max} values for bupivacaine metabolism into PPX were, respectively, 125 μM and 4.78 nmol/min/mg of microsomal protein (Fig. 1).

First, we performed inhibition studies using specific CYP substrates or inhibitors. Caffeine (CYP1A2), coumarin (CYP2A6), tolbutamide (CYP2C), phenytoin (CYP2C), dextrometorphan (CYP2D6, CYP3A), *p*-nitrophenol (CYP2E1), and troleandomycin (CYP3A) (Koop et al., 1989; Relling et al., 1990; Veronese et al., 1991; Dalet-Beluche et al., 1992; Jacqz-Aigrain et al., 1993; Chang et al., 1994; Miners and Birkett, 1996) were used in this aim (Fig. 2). The strongest inhibition was observed for troleandomycin (−95% at 50 μM). A high concentration of dextromethorphan (625 μM) inhibited the degradation of bupivacaine by 36%. Dextromethorphan is metabolized by both CYP2D6 and CYP3A4 (Jacqz-Aigrain et al., 1993), thus it could inhibit CYP3A catalytic activity toward bupivacaine. A high concentration of tolbutamide exhibited slight inhibitory effects whereas the percentages of inhibition obtained with caffeine, coumarin, *p*-nitrophenol, or phenytoin were less than 15%. These results suggest a major involvement of the CYP3A subfamily in the metabolism of bupivacaine in HL microsomes.

Second, the metabolism of bupivacaine into PPX in six HL microsomes was studied to correlate these data with a CYP3A marker activity (Fig. 3). The rate of PPX formation strongly correlated with erythromycin *N*-demethylase activity, with an R value of 0.99. These data also suggest that degradation of bupivacaine into PPX is mainly catalyzed by CYP3A.

In addition, we performed an immunoinhibition study using an anti-rabbit CYP3A6 antibody. The highest concentration of the anti-rabbit CYP3A6 antibody inhibited bupivacaine degradation into PPX by 99% (Fig. 4). These data confirmed the key role of CYP3A in bupivacaine degradation.

To confirm our results, we checked the ability of several cDNA expressed forms of human CYP (CYP1A2, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) to catalyze the biotransformation of bupivacaine into PPX. CYP1A2 and CYP2E1 did not catalyze PPX formation. CYP2C19 and CYP2D6 metabolized bupivacaine into PPX slightly; their turnover numbers were, respectively, 71.2 ± 3.3 pmol/min/nmol of CYP2C19 and 103 ± 4.1 pmol/min/nmol of CYP2D6. CYP3A4 gave the highest turnover number, 931 ± 15 pmol/min/nmol of CYP3A4. CYP3A4 is largely expressed in HL as compared with CYP2C19 and CYP2D6 (Shimada et al., 1994). Taken together, these results were in agreement with the data presented above and provided additional evidence for the involvement of CYP3A4 in the metabolism of bupivacaine.

Although PPX is the major metabolite that has been characterized to date, it nonetheless constitutes only 5% of the dose (Pere et al.,

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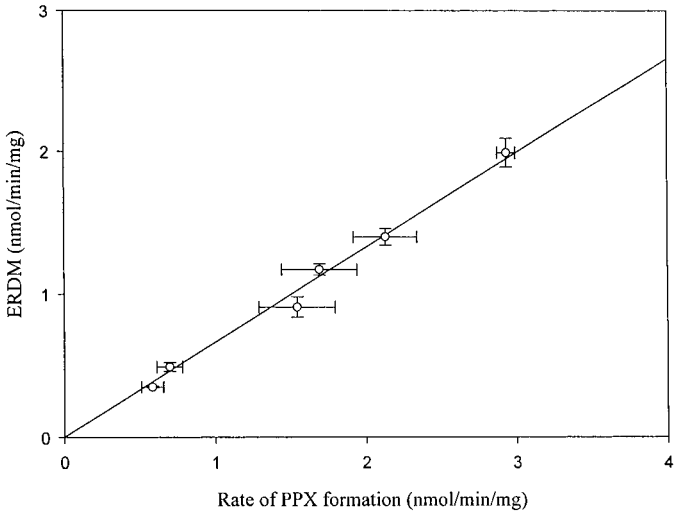

FIG. 3. *Correlation between ERMD activity and the rate of PPX formation for the six samples of HL microsomes used in this study.*

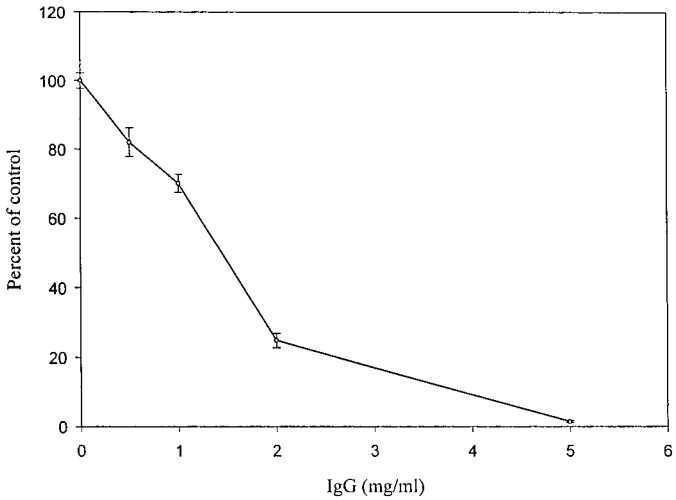

FIG. 4. *Immunoinhibition of bupivacaine oxidative metabolism by anti-CYP3A6 antibodies.*

1991; Rosenberg et al., 1991). Therefore, drug interactions related to CYP3A inhibition seem to be unlikely.

In conclusion, our results demonstrated that CYP3A4 was the main human CYP isozyme catalyzing bupivacaine degradation into PPX, although CYP2C19 and CYP2D6 were also slightly involved in this metabolic pathway. Although bupivacaine is frequently associated with other drugs, an inhibition of CYP3A4 catalytic activity remained unlikely because PPX is only a minor route of biotransformation.

Acknowledgments. We thank Pr. Y. Barra and Dr. E Sérée (UPRESS A Centre National de la Recherche Scientifique 6032, School of Pharmacy, Marseilles, France) for kindly providing us an anti-rabbit CYP3A6 antibody and samples of HL microsomes, and P. Tucker for reviewing the English text.

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