Studies on Cytochrome P450 Responsible for Oxidative Metabolism of Imipramine in Human Liver Microsomes

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The activity of imipramine 2-hydroxylase highly correlated with that of desipramine 2-hydroxylase but not with that of desipramine N-demethylase. The correlation was also found between N-demethylation and 2-hydroxylation when imipramine was used as a substrate, whereas no correlation was observed between them when desipramine was used in place of imipramine. Both activities of desipramine and imipramine 2-hydroxylase were markedly inhibited by quinine but not by quinidine. Although the activity of imipramine N-demethylase was slightly inhibited by both quinidine and quinine, the activity of desipramine N-demethylase was unaffected under the same conditions. The activity of imipramine N-demethylase was roughly correlated with the amounts of P450 3A4 immunochemically determined and the activities of testosterone 6β-hydroxylase in human liver microsomes. The P450 3A4 catalyzed imipramine N-demethylation much more efficiently than 2-hydroxylation in a reconstituted system, whereas neither N-demethylation nor 2-hydroxylation of desipramine was catalyzed by P450 3A4. The activity of imipramine N-demethylation was inhibited, to various extents, by anti-P450 3A4 antibodies in human liver microsomes.

Taking together these and other results, it is suggested that P450 3A4, other than P450 2Cmp, also partly contributes to N-demethylation of imipramine, depending on human liver microsomes.

Keywords cytochrome P450; imipramine; 3A subfamily; human; liver; microsomes

Cytochrome P450 enzymes are ubiquitous membrane-bound heme proteins involved in oxidative metabolism of a wide variety of drugs and carcinogens as well as naturally occurring substances such as steroids, fatty acids and prostaglandins.1,2) It has been known that at least four distinct cytochrome P450 gene families are expressed in the liver of mammalian species including human. The P450 1 family includes genes (P450 1A1 and P450 1A2) that are inducible by polycyclic aromatic hydrocarbon. The P450 2 family is the largest and includes at least five subfamilies: 2A; 2B (phenobarbital-inducible); 2C (constitutively expressed); 2D (associated with debrisoquine 4-hydroxylase); and 2E (ethanol-inducible). The P450 3 family includes genes regulated by pregnenolone 16α-carbonitrile, and the P450 4 family includes genes (involved in fatty acid ω-hydroxylation) that are inducible by clofibrate.3)

It is becoming increasingly clear that cytochrome P450 enzymes from liver microsomes are directly involved in a number of drug-induced toxicities and drug interactions. Some of these adverse effects can be explained in terms of genetic polymorphism; thus, poor metabolizers of debrisoquine are subjected to greater exposure to certain drugs, which may result in either an exaggerated pharmacological effect or an adverse drug reaction.4) Other adverse effects arise from interactions that are likely to occur between two drugs if 1) both drugs are specifically metabolized by the same form of cytochrome P450 or 2) one of them is a specific inducer or a specific inhibitor of the form of cytochrome P450 involved in the metabolism of the other. It is, therefore, evident that in order to predict and/or avoid drug interaction, identification of the form of cytochrome P450 responsible for the metabolism of drugs in question is important.

It has been shown that there was a large interindividual difference in steady-state levels of tricyclic antidepressant drugs.5,6) The variation in the rates of hydroxylation of certain tricyclic antidepressant drugs, such as desipramine, has been demonstrated to be related to the pharmacogenetic defect in the hydroxylation of debrisoquine (P450 2D6).7–9) Imipramine is also eliminated almost exclusively by metabolism. Thus, imipramine is N-demethylated to the active metabolites desipramine and hydroxylated to 2-hydroxymyipramine.10) From the clinical study, the rate of this oxidative metabolism of imipramine is considered to differ greatly between individuals.11) The sparteine/debrisoquine poor metabolizers have been demonstrated also to be poor hydroxylators of imipramine,12) indicating that P450 2D6 may be involved in 2-hydroxylation of imipramine. In contrast to 2-hydroxylation, although multiple forms of cytochrome P450 have been suggested to be responsible for N-demethylation of imipramine,13–16) the cytochrome P450 involved in N-demethylation of imipramine has not yet been fully identified. The purpose of this research was to gain a better understanding of the properties of N-demethylation of imipramine in human liver microsomes.

Materials and Methods

Materials NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast, Tokyo, Japan. Testosterone metabolites and Emulgen 911 were generous gifts from Dr. Kirk, Queen Mary College, University of London, and Kao-Atlas, Tokyo, Japan, respectively. Sepharose 4B and protein A Sepharose (Fast Flow) were obtained from Pharmacia, Uppsala, Sweden. Other chemicals used were of the highest grade commercially available. A preparative DEAE-SPW and hydroxylapatite columns were purchased from Tosoh, Tokyo, Japan and Koken, Tokyo, Japan, respectively. Liver autopsy samples were obtained within 20 h after death, and stored at −80°C until use. Microsomes were prepared as described elsewhere.17)

Monooxygenases Assays Reaction mixture consisted of 100 mM
potassium phosphate (pH 7.4), 0.1 mM EDTA, NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 0.1 unit of glucose 6-phosphate dehydrogenase and 6 mM MgCl₂), microsomal protein (approx. 1.0 mg) and substrate in a final volume of 1.0 ml. If necessary, various agents were added to the reaction mixture at concentrations specified in the figures and the legends of figures. The reaction was started by the addition of a NADPH-generating system which had been preincubated at 37°C for 3 min. The incubations were carried out at 37°C for 10 min aerobically, and terminated by the addition of 1 ml of 1 M carbonate buffer (pH 10.0). After internal standard was added, the ethylacetate extract was evaporated to dryness under reduced pressure. Nortriptyline and amitriptyline were used as internal standards for determination of imipramine and desipramine metabolites, respectively. Analysis of imipramine, desipramine and their metabolites was carried out by high-performance liquid chromatograph (Hitachi model L-6000) equipped with a UV absorbance detector (Hitachi model L-4200). For detection of metabolites, reverse-phase chromatography was performed on a Shiseido Capcell Pak C18 column (4.6×250 mm). The metabolites were separated at a flow rate of 1 ml/min and by an isocratic elution (methanol:acetonitrile:water = 53:44:2, pH 9.3). The effluent was monitored at 254 nm. Testosterone hydroxylase activities were measured according to the method of Hayashi et al.18

**Inhibition of Monoxygenase Activities by Anti-P450 Antibodies and Triacylloleandomycin**

Liver microsomes were incubated 10 min at 37°C in the presence of anti-P450 3A4 antibodies. After substrate and NADPH-generating system were added to the medium, the reaction was carried out as indicated in the previous section. Triacylloleandomycin (100 mM) was added to the reaction mixture without substrate, and then preincubation was carried out for 15 min at 37°C with shaking.

**Purification of P450 3A4 and NADPH-Cytochrome P450 Reductase and Reconstitution of Monoxygenase System**
P450 3A4 was purified to electrophoretic homogeneity from human liver microsomes according to the method described elsewhere.19 NADPH-cytochrome P450 reductase and cytochrome b₅ were purified from liver microsomes of phenobarbital-treated rats according to the methods of Yasukoch and Masters, and Imai, respectively, with minor modifications.20,21 The reconstituted system consisted of 0.1 nmol of P450 3A4, 0.15 mM microsomal lipid, 10 pmol of cytochrome b₅, 1 unit of NADPH-cytochrome P450 reductase, and other components described above in a final volume of 1 ml. The reaction was carried out for 15 min at 37°C.

**SDS-PAGE and Immunoblot**
Electrophoretic analysis of microsomes was carried out with 10% polyacrylamide gel according to the method of Laemmli.22 Immunoblot of microsomes was carried out as previously reported.23 The microsomal proteins separated on polyacrylamide gel were electrophoretically transferred to nitrocellulose filters (Schleicher & Schnell, Dassel, Germany). Staining of the blot was carried out with appropriate peroxidase-labeled species specific antibody, diaminobenzidine being used as the substrate. Relative concentrations of cytochrome P450 purified out the various microsomal preparations were estimated from densitometric analysis of the blots with Shimazu dual-wavelength scanner.

**Other Methods**
Microsomal protein was measured according to the method of Lowry et al.24 using bovine serum albumin as the standard. Cytochrome P450 was determined by the method described elsewhere.25 The activity of NADPH-cytochrome c (cytochrome P450) reductase was measured according to the method of Phillips and Langdon.26 One unit of the reductase activity was defined as the amount of enzyme catalyzing the reduction of cytochrome c at a rate of 1 μmol per min. Microsomal lipids were extracted from intact rat liver microsomes by the method of Folch et al.27 The g-globulin fraction of rabbit antisem was prepared using protein A Sepharose, according to the protocol provided by the supplier.

**Results**
Table I shows the relationship of oxidative metabolism between imipramine and desipramine in human liver microsomes. As expected from the observations reported previously,13,15 the activity of imipramine 2-hydroxylase highly correlated with that of desipramine 2-hydroxylase but not with that of desipramine N-demethylase in human liver microsomes. In contrast, the activity of imipramine N-demethylyase did not correlate with that of desipramine N-demethylyase. The significant correlation was found between N-demethylylation and 2-hydroxylation when imipramine was used as substrate, whereas no correlation was observed between them when desipramine was used in place of imipramine. In addition, the activity of imipramine N-demethylyase was found to correlate significantly with that of desipramine 2-hydroxylase. These result indicate that imipramine 2-hydroxylase may be mainly catalyzed by the same enzyme which is involved in desipramine 2-hydroxylation, whereas cytochrome P450 responsible for imipramine N-demethylylation is not necessarily the same enzyme responsible for desipramine N-demethylylation.

As shown in Fig. 1, quinidine, which is known to be one of the specific inhibitors of P450 2D, markedly inhibited the activity of desipramine 2-hydroxylase by about 80% when compared to control value, whereas quinine showed only a weak inhibitory effect on the activity. In contrast, the activity of desipramine N-demethylase was insensitive against quinidine at the concentration of 5 μM. On the other hand, the activity of imipramine 2-hydroxylase was also markedly inhibited by quinidine but not by quinine. Both quinidine and quinine weakly but reproducibly inhibited the activity of imipramine N-demethylyase. These results indicate that P450 2D is one of the major forms of cytochrome P450 responsible for 2-hydroxylation of imipramine in human liver microsomes as in the case of desipramine. It is also suggested that P450 2D catalyzes, at least in part, N-demethylation of imipramine, although the form(s) of cytochrome P450 other than P450 2D can be
considered to contribute to N-demethylation of imipramine in human liver microsomes.

The addition of propranolol resulted in the decrease in the activities of both 2-hydroxylase and N-demethylase of imipramine (Fig. 2). The extent of inhibition by propranolol of 2-hydroxylase activity was, however, somewhat different from that observed in N-demethylation. The activity of 2-hydroxylase in liver microsomes was inhibited by propranolol in a dose-dependent manner, and was inhibited by 85-90% when compared to control, whereas the maximal inhibition of N-demethylation was by 50-60% of control in the same conditions. Since P450 2D and P450 2C have been demonstrated to be involved in the oxidative metabolism of propranolol,^26^ these results suggest the contribution of P450 2C and/or P450 2D to N-demethylation of imipramine in human liver microsomes. Furthermore, judging from the maximal inhibition of imipramine N-demethylation by propranolol, it seemed possible that cytochrome P450 other than P450 2C and/or P450 2D may also contribute to the reaction.

Since quinidine has been shown to be not only a strong inhibitor against P450 2D6 but also a substrate for P450 3A4,^26^ the relationship among the activities of imipramine N-demethylase and testosterone 6β-hydroxylase, and the amount of P450 3A4 was investigated (Fig. 3). The activities of N-demethylase of imipramine appeared to be associated with the activities of 6β-hydroxylase of testosterone and the levels of P450 3A4 in human liver microsomes. As expected, the activity of testosterone 6β-hydroxylase, which is known to be classified in the cytochrome P450 3 family, showed a good correlation with the amounts of P450 3A4 immunochemically determined in human liver microsomes ($r^2 = 0.65$, $n = 28$, $p < 0.01$). The activity of imipramine N-demethylase was also correlated with both the activity of testosterone 6β-hydroxylase ($r^2 = 0.65$, $n = 28$, $p < 0.01$) and the amounts of P450 3A4 ($r^2 = 0.65$, $n = 28$, $p < 0.01$) in human liver microsomes. In addition, in agreement with the observations reported previously,^14,26^ the activity of N-demethylase of imipramine correlated with the amounts of P450 2Cmp ($r^2 = 0.24$, $n = 28$, $p < 0.05$) and P450 2D6 ($r^2 = 0.32$, $n = 28$, $p < 0.01$) in human liver microsomes.

![Fig. 2. Inhibition by Propranolol of N-Demethylation and 2-Hydroxylation of Imipramine in Human Liver Microsomes](image)

The activities of imipramine N-demethylase and 2-hydroxylase were measured in the presence of various concentrations of propranolol as specified in the figure. The concentrations of imipramine used were 20 μM (■) and 100 μM (○), respectively. Each value represents the mean of duplicate determinations.

![Fig. 3. Relationship of N-Demethylation of Imipramine with 6β-Hydroxylation of Testosterone and Content of Immunochemically Determined P450 3A4 in Human Liver Microsomes](image)

The measurement of the activities of imipramine N-demethylase (A) and testosterone 6β-hydroxylase (B), and immunoblots (C) were carried out as described in Materials and Methods.

![Fig. 4. Interindividual Differences in the Inhibitory Effects of Triacetylethandamine on N-Demethylation of Imipramine in Human Liver Microsomes](image)

Pretreatment of microsomes with triacetylethandamine and the assay for N-demethylation of imipramine were carried out as described in Materials and Methods. The activities of imipramine N-demethylase in human liver microsomes without pretreatment were as follows: No. 6, 240 pmol/mg/min; No. 14, 319 pmol/mg/min; No. 15, 173 pmol/mg/min; No. 16, 494 pmol/mg/min.
The inhibition of 2-hydroxylation by anti-P450 3A4 antibodies was less than 10% in human liver microsomes (data not shown).

Discussion
In the present study, we examined the oxidative metabolism of imipramine by human liver microsomes. In agreement with the results of in vivo and in vitro studies by Broesen et al.\textsuperscript{13,15} who showed that 2-hydroxylation of imipramine and desipramine depended almost exclusively on the sparteine/debrisoquine oxygenase (P450 2D6), the activity of 2-hydroxylase of imipramine was highly correlated with that of desipramine in human liver microsomes, and the 2-hydroxylation of imipramine was almost completely inhibited by quinidine but not by quinine. From these results, it is concluded that P450 2D6 plays a major role for 2-hydroxylation of imipramine in human liver microsomes. On the other hand, although N-demethylation of imipramine was inhibited by quinidine, the inhibition was much less than that observed in 2-hydroxylation, suggesting that the contribution of P450 2D6 to N-demethylation of imipramine in human liver microsomes may be very little, if any. The reason why N-demethylation of imipramine correlated with 2-hydroxylations of imipramine and desipramine cannot be explained at present. More recently, it has been suggested from clinical study that P450 2Cmp is partly involved in N-demethylation of imipramine.\textsuperscript{14} Propranolol, which has been shown to be metabolized by P450 2Cmp and P450 2D6,\textsuperscript{29,30} inhibited both 2-hydroxylation and N-demethylation of imipramine in liver microsomes. However, the maximal inhibition by propranolol of imipramine N-demethylation was not more than 60% of control value. P450 3A4 was capable of metabolizing imipramine to desipramine in a reconstituted system, and the anti-P450 3A4 antibodies inhibited N-demethylation of imipramine depending on the liver microsomes used. In addition, the activity of N-demethylase of imipramine was correlated with the activity of 6β-hydroxylation of testosterone and the amounts of P450 3A4, indicating that P450 3A4 may also be, at least in part, responsible for N-demethylation of imipramine in human liver microsomes. The levels of P450 3A4 have been shown to be induced in humans by administration of macrolides and glucocorticoids.\textsuperscript{31} In addition, it has been shown that cytochrome P450 belonging to the human P450 3 family was polymorphically expressed in human livers.\textsuperscript{32} Whether the individual differences in the oxidative metabolism of imipramine in vivo can be, in part, accounted for by the levels of P450 3A4 and/or a polymorphically expressed member of the P450 3 family remains to be investigated.

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References