Contribution of Flavin-Containing Monooxygenase and Cytochrome P450 to Imipramine N-Oxidation in Rat Hepatic Microsomes

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Enzymatic formation of desipramine (DMI) and imipramine N-oxide (IMINO) was kinetically characterized in rat liver microsomes at pH 8.5 and 7.5. The formation of IMINO was quickly suppressed by the preincubation of microsomes at 37°C at pH 8.5, but the suppression was comparatively gentle at pH 7.5. In kinetic studies, the formation of DMI was monophasic at the two pH points, and a substrate inhibition was observed at pH 8.5, but not at pH 7.5. In contrast, the formation of IMINO was biphasic at both pH points, i.e., the summation of a low-Km phase and a high-Km phase. Methimazole (MZ), an inhibitor of flavin-containing monooxygenase (FMO), markedly suppressed the low-Km phase of IMINO formation at both pH points. MZ also suppressed DMI formation at pH 8.5, but it elevated DMI formation at pH 7.5. SKF 525-A, an inhibitor of cytochrome P450 (CYP), markedly suppressed DMI formation at both pH points. The inhibitor suppressed IMINO formation in the high-Km phase of the biphasic kinetics at both pH points, whereas it stimulated the activity of the low-Km phase at pH 7.5. These results suggest that CYP enzyme(s) are mainly responsible for DMI formation at pH 8.5 and 7.5, and FMO enzyme(s) also are involved in IMI N-demethylation at a higher pH range in rat liver microsomes, at least in part. In the formation of IMINO, FMO is a major enzyme at both pH points, and CYP may also contribute to the N-oxide formation to some extent at pH 8.5.

Key words flavin-containing monooxygenase; cytochrome P450; rat liver microsome; enzyme kinetics; methimazole; SKF 525-A

Imipramine (IMI) is widely prescribed for the treatment of the major depression. 1) After oral administration, IMI is easily absorbed from the intestinal wall and converted to several oxidative metabolites in the liver. Desmethylimipramine (desipramine, DMI) (Fig. 1) is one of the major IMI metabolites formed by cytochrome P450 (CYP),2–4) and is pharmacologically equipotent to the parent compound, so that DMI is thought to contribute to the antidepressant effect of IMI as an active metabolite. 1,3) IMI and DMI are oxidized at the 2-position by CYP, forming 2-hydroxylated metabolites with less pharmacological activity. 5–7)

IMI N-oxide (IMINO, Fig. 1) is formed as another active metabolite which has an antidepressant activity similar to that of the parent compound. 8–10) It has been reported that flavin-containing monooxygenase (FMO) is the major enzyme responsible for the formation of IMINO. 11) However, there is also a possibility that CYP mediates the formation of IMINO. In general, FMO is relatively thermolabile and has a higher optimal pH in the reaction compared to the reactions mediated by CYP. 12) Therefore, the contribution of these enzymes to some reaction can be altered by the experimental conditions employed, such as pH and preincubation time.

In the present experiments, we examined to what extent FMO and CYP contribute to the N-oxidation of IMI using enzyme inhibitors or inactivation techniques.

MATERIALS AND METHODS

Chemicals IMI and DMI hydrochlorides were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), IMINO and 10-hydroxydesipramine (10-OH-DMI) were kindly supplied by Ciba-Geigy Co., Ltd. (Basel, Switzerland). NADPH and glucose 6-phosphate were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Methimazole (MZ) and SKF 525-A were obtained from Sigma Chemical Co. Other reagents used were of the highest grade commercially available.

Enzyme Sources Adult male Wistar rats (6–7 weeks old) were obtained from Takasugi Experimental Animals Lab. (Saitama, Japan). The animals were kept in animal rooms at 22–24°C with a 12 h light and dark cycle for at least one week prior to the experiments. The rats were sacrificed, then livers were perfused with 0.9% NaCl to remove blood. Microsomal fractions of livers and brains were prepared by a published method. 13)

Enzyme Assay A typical reaction mixture (final volume of 1.0 ml, pH 7.5 or 8.5) consisted of microsomes (1 mg protein), NADPH (1.25 μmol) and Tris–HCl buffer (150 μmol) in a brown conical glass tube with a stopper (10 ml volume). The mixture was preincubated at 37°C for 5 min, then IMI HCl (2.5 μM–5 mM) was added and incubated at 37°C for 1 min. At the end of the incubation, 1.0 ml of 1 N NaOH aqueous solution was added as a reaction stopper and 1 nmol of 10-OH-DMI as an internal standard. Ethyl acetate (6 ml) was then added, and the tube was vigorously shaken and centrifuged (1500 × g for 10 min). The organic layer (5 ml) was taken, evaporated to dryness, and the residue was dissolved in 100 μl of the mobile phase of high performance liquid chromatography (HPLC) as described below. IMINO and DMI were determined by the internal standard method on the basis of the calibration curve. The calibration curve was made up by adding known amounts of synthetic IMINO, DMI and 1 nmol of 10-OH-DMI to ice-cold incubation medium containing various ingredients.

HPLC Conditions The HPLC system consisted of a Shimadzu LC-3A pump, a Rheodyne 7125 injector and a Shimadzu SPD-2A spectrophotometric detector. Other conditions were: column, Li Chrospher Si 60 (Meerk, Darmstadt, Germany); mobile phase, CH3CN–MeOH=29% NH3-571 (1999)}
(72:28:4, by volume); flow rate, 1.5 ml/min; detection, UV 254 nm.

Others Protein concentrations were determined by the method of Lowry et al. with bovine serum albumin as a standard. Enzyme kinetic parameters (Km and Vmax) were analyzed according to a non-linear least-square regression analysis based on a simplex method. Statistical significance was calculated by Student's t-test.

RESULTS AND DISCUSSION

In previous experiments, we compared the enzymatic formation of IMINO from IMI between hepatic and brain microsomes in the rat, and confirmed that FMO is the major enzyme in the formation of IMINO in the two microsomal fractions at pH 9.0 of the incubation medium. In the present study, we have examined the formation of IMINO and DMI from IMI at various pH points from 7.0 to 10.0 using HPLC conditions which were slightly different from the previous ones. As shown in Fig. 1, three peaks of DMI (6.2 min), 10-OH-DMI as an internal standard (8.4 min) and IMINO (10.1 min) were sufficiently separated on a chromatogram of HPLC.

We used the incubation time of 1 min at 37°C during which no peak corresponding to biologically formed 10-OH-DMI was found in preliminary experiments. The activity for IMINO formation gradually increased along with the elevation of pH, and showed a small peak at round pH 9.5 (Fig. 2). In contrast, the DMI forming activity showed a peak at pH 7.5 and another small peak at around pH 9.5. From these results, we employed two pH points (7.5 and 8.5) in further experiments, because pH 9.0 or above was thought to be too far from the physiological conditions in the liver.

Thermal stability is a characteristic property of FMO. We tested the time course of change in the formation activities of IMINO and DMI under different pH conditions. The activity for IMINO formation at pH 8.5 decreased to about 10% after 5 min preincubation without NADPH as a cofactor, whereas that at pH 7.5 did not decrease so quickly, and about 20% of the activity remained until 15 min of the preincubation without the cofactor (Fig. 3). In contrast, the activity for DMI formation showed only a slight decrease (about 15%), even after 15 min preincubation at 37°C without NADPH. We thus chose the preincubation time of 15 min at 37°C, and examined the significance of the change in the formation of DMI and IMINO (Fig. 4A). At pH 8.5, about 90% of IMINO forming activity was impaired as compared with the control without the preincubation, whereas DMI forming activity was not different between the treated and the control groups. At pH 7.5, a similar result was obtained, but the remaining activity of IMINO formation was 26% of the control after the preincubation. These results, together with those in Fig. 3, suggest that the participation of the thermolabile enzyme in IMINO formation is larger at pH 8.5 than at pH 7.5. In other words, it is possible that some enzyme (maybe CYP) that is not so sensitive as FMO to the preincubation at 37°C is involved in the formation of IMINO in the reaction at pH 7.5.

We then conducted enzyme kinetics using substrate concentrations ranging from 2.5 μM to 5 mM. Typical Eadie-Hofstee plots for the formation of DMI and IMINO in the control and inhibition experiments are shown in Fig. 5. In Eadie-Hofstee plots, substrate inhibition was observed in the DMI formation at pH 8.5 (Fig. 5A, open symbols), whereas the plots for the IMINO formation (Fig. 5B, open symbols) were biphasic. In contrast, no substrate inhibition was observed in the formation of DMI at pH 7.5, as shown in Eadie-Hofstee plots (Fig. 5C, open symbols), which were analyzed to be monophasic. IMINO formation at pH 7.5 was biphasic (Fig. 5D, open symbols), which is similar to the profile at pH 8.5 (Fig. 5B, open symbols). These results were reproducible in two more experiments using different lots of microsomal fractions.

Fig. 1. A Typical Chromatogram of Ethyl Acetate Extract
IMI (1 ms) was incubated with rat liver microsomes in the presence of NADPH at 37°C for 1 min, and the metabolites formed were extracted with ethyl acetate. HPLC conditions are given in Materials and Methods. Retention times of DMI, 10-OH-DMI (internal standard) and IMINO are 6.2, 8.4, and 10.1 min, respectively.

Fig. 2. Change in the Formation of DMI and IMINO at Various pH Points of the Reaction Medium Containing Rat Liver Microsomes
IMI (1 ms) was incubated with rat liver microsomes (1 mg/ml protein) and NADPH (1.25 ms) in the reaction medium containing 150 ms Tris-HCl buffers of pH 7.0 to 10.0. Other conditions are given in Materials and Methods. Open circle, DMI; closed circle, IMINO; each point is the mean value of duplicate determinations.

Hofstee plots, substrate inhibition was observed in the DMI formation at pH 8.5 (Fig. 5A, open symbols), whereas the plots for the IMINO formation (Fig. 5B, open symbols) were biphasic. In contrast, no substrate inhibition was observed in the formation of DMI at pH 7.5, as shown in Eadie-Hofstee plots (Fig. 5C, open symbols), which were analyzed to be monophasic. IMINO formation at pH 7.5 was biphasic (Fig. 5D, open symbols), which is similar to the profile at pH 8.5 (Fig. 5B, open symbols). These results were reproducible in two more experiments using different lots of microsomal fractions.
Fig. 3. Effects of Preincubation at 37 °C on the Formation of DMI and IMINO from IMI in Rat Liver Microsomes

Rat liver microsomes (1 mg protein) were preincubated without NADPH in the reaction medium (pH 8.5 or 7.5) at 37 °C for 0, 5, 10 and 15 min, followed by the addition of the substrate (IMI, 1 mM) and NADPH (1.25 mM) and incubation for 1 min. Other conditions are given in Materials and Methods. Closed circle, IMINO formation; open circle, DMI formation. Each point is the mean value of duplicate determinations. Control DMI-forming activities were 2.539 and 1.751 nmol/min/mg protein for pH 7.5 and 8.5, respectively; control IMINO-forming activities were 0.571 and 1.025 nmol/min/mg protein, respectively.

Fig. 4. Effects of Preincubation at 37 °C (A), MZ (B) and SKF 525-A (C) on the Formation of DMI and IMINO from IMI in Rat Liver Microsomes

(A) Rat liver microsomes (1 mg protein) were preincubated at 37 °C for 15 min in the reaction medium of pH 8.5 or 7.5, followed by the addition of IMI (1 mM) and NADPH (1.25 mM) and incubation at 37 °C for 1 min. (B) MZ (1 mM) or (C) SKF 525-A (1 mM) was added to the incubation medium (pH 8.5 or 7.5) containing rat liver microsomes (1 mg protein) and NADPH (1.25 mM), and preincubated at 37 °C for 5 min. IMI (5 μM or 1 mM) was then added to the medium, and the mixture was incubated at 37 °C for 1 min. The values are expressed as a percent of the control (100%). Dotted columns, DMI; hatched columns, IMINO. Control DMI-forming activities at pH 8.5 were 0.239±0.033 (5 μM) and 1.687±0.203 (1 mM) nmol/min/mg protein and those at pH 7.5 were 0.314±0.052 (5 μM) and 2.205±0.190 (1 mM) nmol/min/mg protein. Control IMINO-forming activities at pH 8.5 were 0.109±0.015 (5 μM) and 1.119±0.194 (1 mM) nmol/min/mg protein and those at pH 7.5 were 0.038±0.044 (5 μM) and 0.659±0.061 (1 mM) nmol/min/mg protein. Each value in the figure represents relative activity±S.D. (n=4). *p<0.05; **p<0.01.

Calculated kinetic parameters are summarized in Table 1. The formation of IMINO was analyzed to be biphasic, i.e., the summation of a low- Km phase and a high-Km phase at each pH condition. Michaelis constant (Km) and maximal velocity (Vmax) values for the high-Km phase were very similar between pH 8.5 and 7.5. However, the Km and Vmax values of the low-Km phase tended to increase and decrease, respectively, at pH 7.5, resulting in the clearance value (Vmax/Km) at pH 7.5 being one-third that at pH 8.5. In calculating the kinetic parameters for the formation of DMI at pH 8.5, the data in a higher substrate concentration range (0.5 to 5 mM) showing substrate inhibition were neglected. The data suggest that Km values are similar between pH 8.5 and 7.5, whereas Vmax values at pH 8.5 seem to be larger than those at pH 7.5, resulting in 5-fold different clearance values (pH 8.5>pH 7.5).

It is unclear at present what causes the substrate inhibition in the formation of DMI from IMI at pH 8.5, but it is noteworthy that the Vmax values calculated from the data, from which the data in the substrate concentration range (0.5 to 5 mM) showing the substrate inhibition were omitted, were much larger than those at pH 7.5.

To determine the contribution of FMO and CYP enzymes to the formation of DMI and IMINO, we examined the effects of two enzyme inhibitors on their activities. MZ is a well-known inhibitor of FMO. We added the inhibitor (final concentration 1 mM) to the incubation medium and it was preincubated with NADPH at 37 °C for 5 min, followed by incubation with the substrate (5 μM or 1 mM). As shown in Fig. 4B, the formation of IMINO at pH 8.5 and pH 7.5 (hatched columns) was significantly suppressed by the addition of MZ compared to that of the control without the inhibitor. However, the formation of DMI (dotted columns) was affected biphasically; i.e., DMI formation at pH 8.5 was significantly suppressed by MZ, whereas at pH 7.5 tended to increase in the presence of MZ as compared with the control incubation without the inhibitor. The formation of IMINO seems more sensitive to MZ than the formation of DMI.

SKF 525-A is a typical but nonspecific inhibitor of CYP. The formation of DMI was markedly suppressed by SKF.
525-A (final concentration 1 mM) at the substrate concentrations of 5 μM and 1 mM in the incubation medium of pH 8.5 and 7.5 (Fig. 4C). In contrast, IMINO formation was weakly affected compared to DMI formation under any of the conditions used, and the IMINO forming activity was significantly higher than the control value at a substrate concentration of 5 μM at pH 8.5. In further experiments, we kinetically examined the effects of the inhibitors on the formation of DMI and IMINO.

Figure 5 (A, B, C and D) summarizes the effects of MZ at pH 8.5 and 7.5. As shown in Eadie-Hofstee plots (Fig. 5B and D, closed symbols), MZ selectively inhibited the activity of the low-<i>K<sub>m</sub></i> phase, and the high-<i>K<sub>m</sub></i> phase was not affected in the biphasic kinetics of IMINO formation. On the other hand, DMI formation at pH 8.5 was markedly suppressed by MZ (Fig. 5A, closed symbols), whereas that at pH 7.5 was inversely stimulated (Fig. 5C, closed symbols). This stimulation is thought to reflect the elevated activities of DMI formation in Fig. 4B at pH 7.5.

Figure 5 (E, F, G and H) shows the effects of SKF 525-A under the two pH conditions. As expected from the results of Fig. 4C, DMI formation was suppressed by the inhibitor at both pH points of 8.5 and 7.5 (Fig. 5E and G, closed symbols). Interestingly, IMINO formation, particularly in the high-<i>K<sub>m</sub></i> phase, was suppressed by SKF 525-A at pH 8.5 (Fig. 5F, closed symbols). In the N-oxide formation at pH 7.5 (Fig. 5H), the activity in the low-<i>K<sub>m</sub></i> phase seemed to be slightly stimulated compared to the profile of the control without the inhibitor, whereas the activity in the high-<i>K<sub>m</sub></i> phase seemed to be suppressed to some extent by the inhibitor.

The formation of IMINO involved biphasic kinetics; the low-<i>K<sub>m</sub></i> phase was markedly suppressed by a typical FMO inhibitor, MZ, but the high-<i>K<sub>m</sub></i> phase was not affected in the incubation mixtures of pH 8.5 and 7.5. This result suggests that IMINO formation at pH 8.5 and 7.5 is mediated by both FMO as low-<i>K<sub>m</sub></i> enzyme(s) and CYP as high-<i>K<sub>m</sub></i> enzyme(s), although the contribution of the FMO enzyme(s) to the IMINO formation is much higher than that of the CYP en-
zyme(s).

The formation of DMI was markedly suppressed by SKF 525-A, indicating that CYP enzyme(s) are mainly responsible for IMI N-demethylation at pH 8.5 and 7.5, as reported previously.2-4 It is of interest that the DMI formation was also suppressed by MZ at pH 8.5, suggesting that FMO enzyme(s) also are involved at least in part in DMI formation from IMI at a higher pH range in rat liver microsomes. Guo et al.10 recently reported that MZ is not a specific FMO inhibitor, but that it inhibits some CYP enzymes in human liver microsomes. This knowledge raises the possibility that MZ nonspecifically inhibits some CYP enzymes responsible for IMI N-demethylation in rat liver microsomes.

SKF 525-A tended to suppress the IMINO formation at pH 8.5, especially the high-K_m phase. Figure 5H indicates that the addition of the inhibitor biphasically affected the formation of IMINO: i.e., SKF 525-A stimulated IMINO formation in the low-K_m phase, while it inversely suppressed IMINO formation in the high-K_m phase. These complicated effects of the inhibitor might result in the lack of effect of SKF 525-A on IMINO formation at pH 7.5 (Fig. 4C). Together with the selective suppression by MZ of the low-K_m phase of IMINO formation at pH 8.5, this result suggests that besides FMO as a major enzyme, CYP also contributes to some extent to the N-oxide formation at pH 8.5. We do not have concrete evidence to explain why MZ and SKF 525-A showed stimulation of the formation of DMI and IMINO, respectively, at pH 7.5. Since both FMO and CYP use NADPH as a cofactor, it is possible that the inhibition of the main enzyme(s) by the inhibitor may increase the availability of the cofactor for the other enzyme(s) which are not affected by the inhibitor. However, this cannot explain the fact that no stimulation was observed at pH 8.5.

Substrate inhibition was observed only in DMI formation in the present study. To our best knowledge, this is the first report showing substrate inhibition in IMI N-demethylation. From the present inhibition experiments, we think that CYP enzyme(s), but not FMO, are involved as major enzyme(s) in the substrate inhibition. A possible cause for the suppression of the activity might be a shortage of the cofactor (NADPH) supplement for the reactions of CYP and FMO due to higher substrate concentration. However, this seems unlikely because we employed a short incubation time of 1 min to obtain the initial velocity of the reactions in the present study. Furthermore, when we used double or triple amounts of NADPH or an NADPH-generating system, essentially similar results were obtained.

We reported previously that the repetitive oral administration of IMI to rat decreased drug oxidation activities catalyzed by CYP2D enzyme(s) in rat liver microsomes,19 and the enzymes may be inactivated by covalent binding with a possible reactive metabolite formed from IMI (maybe an epoxy metabolite) as a mechanism-based inactivation.20 The suicidal inactivation, however, may not be a cause in the present situation, because the substrate inhibition occurred only at pH 8.5 and not at pH 7.5. Hence, we have to think of other factors such as a possible change in the charge of functional groups of amino acid residues located in the active site of the enzyme(s), resulting in a possible alteration of the interaction between the enzyme(s) and the substrate. Further study is necessary to elucidate the mechanism causing the substrate inhibition.

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