Cytochrome P4502D and -2C Enzymes Catalyze the Oxidative *N*-Demethylation of the Parkinsonism-Inducing Substance 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine in Rat Liver Microsomes

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We have examined the oxidative *N*-demethylation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a Parkinsonism-inducing neurotoxin, in liver microsomes from adult Wistar and Dark Agouti (DA) rats. The oxidation of MPTP to 4-phenyl-1,2,3,6-tetrahydropyridine (PTP) in these preparations required NADPH as a cofactor and was significantly inhibited by SKF 525-A (2 mM). MPTP *N*-demethylation exhibited biphasic kinetics, consistent with two enzymes, a low K_m system (K_{m1}, 10.0 ± 2.2 μM; V_{max1}, 0.048 ± 0.009 nmol/(min·mg of protein)) and a high K_m system (K_{m2}, 1180 ± 91 μM; V_{max2}, 4.80 ± 0.75 nmol/(min·mg of protein)). We thus employed two substrate concentrations, 5 μM and 5 mM, for the low and high K_m system, respectively, to assay enzyme activity in subsequent experiments. The oxidation activity was significantly decreased by pretreatment of rats with phenobarbital and β-naphthoflavone. Furthermore, marked strain (Wistar > DA) and sex (male > female) differences were observed at low (5 μM) and high (5 mM) substrate concentrations, respectively. Reconstitution experiments with cytochrome P450BTL, which belongs to the P4502D subfamily, and P450m1 (P4502C11) demonstrated that MPTP *N*-demethylase occurs at concentrations of 5 μM and 5 mM. At 5 mM the male-specific P450m1 showed a remarkably high activity, over 400-fold that of P450BTL. Polyclonal antibodies against P450BTL and P450m1 effectively suppressed the activity at the low (5 μM) and the high (5 mM) substrate concentrations, respectively. These results suggest that, in the microsomal preparations used, MPTP *N*-demethylation is mainly mediated by P4502D enzyme(s) at lower substrate concentrations and by P4502C11 at higher substrate concentrations.

Introduction

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[1] is a precursor to a Parkinsonism-inducing neurotoxic agent (*1*, *2*). The compound easily passes through the blood−brain barrier and is oxidized to 1-methyl-4-phenyl-2,3-dihydropyridinium ion ($MPDP^+$) by monoamine oxidase B in brain tissues (Figure 1). $MPDP^+$ is then nonenzymatically converted to the toxic compound *N*-methyl-4-phenylpyridinium ion (MPP^+), which, after being taken up into dopaminergic neurons, causes neuronal toxicity in experimental animals and humans (*3*−*5*).

MPTP is also oxidized by the microsomal flavin-containing monooxygenase (FMO) to MPTP *N*-oxide, and this pathway was thought to be the major detoxication pathway (*6*−*8*). Cytochrome P450 (P450) has been reported to be involved in other oxidative pathways of MPTP such as *N*-demethylation to 4-phenyl-1,2,3,6-tetrahydropyridine (PTP) (*6*, *8*), a nontoxic metabolite (*9*), and oxidative dehydrogenation forming $MPDP^+$ and MPP^+ (*10*) (Figure 1). Accumulated knowledge indicates

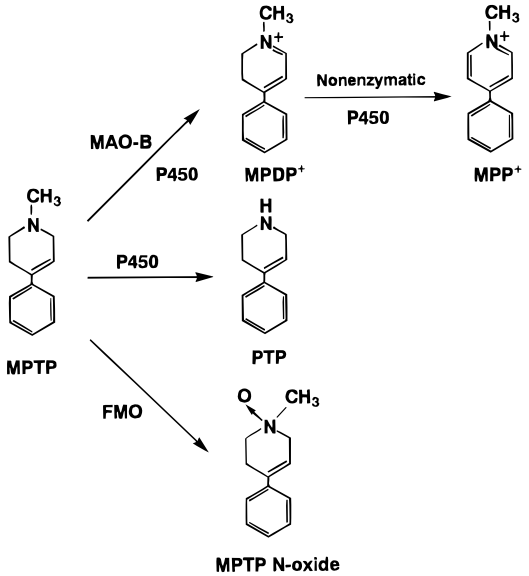

Figure 1. Oxidative metabolism of MPTP in the liver. MAO-B, monoamine oxidase B.

that MPTP is primarily converted to $MPDP^+$ by monoamine oxidase B, and contribution of P450 forming PTP and FMO forming MPTP *N*-oxide to the overall MPTP biotransformation is limited in the peripheral system, especially in the liver (*6*, *7*, *11*). However, the formation

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[1] Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PTP, 4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium ion; MPP^+, *N*-methyl-4-phenylpyridinium ion; FMO, flavin-containing monooxygenase; P450, cytochrome P450; DA, Dark Agouti; SD, Sprague-Dawley; G-6-P, glucose 6-phosphate.

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of PTP is thought to be only one oxidative detoxication pathway of MPTP because after direct administration to mouse brain, MPTP *N*-oxide, which had been believed to be the major detoxicated metabolite, exerted a neurotoxicity similar to MPTP by causing a decrease of dopamine in nigrostriatal neurons (*12*).

There have been several reports on the metabolic interaction between MPTP and P4502D enzymes (*13*−*16*), demonstrating that the compound inhibits P4502D reactions in humans and rodents. Fonne-Pfister *et al*. (*13*, *14*) found that bufuralol 1′-hydroxylase, a reaction mediated by P4502D enzymes, was competitively inhibited by MPTP in microsomal fractions from human liver or rat brain. Among three pathways in which P450 can be involved (Figure 1), the oxidative dehydrogenation pathways forming $MPDP^+$ and MPP^+ have been shown to be mediated by rat liver P4502B1 and P4501A1 in the reconstituted system (*9*). Thus, the formation of PTP from MPTP may be catalyzed by P4502D enzymes. Genetic polymorphism of human P4502D enzyme (2D6) has been suggested to be associated with susceptibility to Parkinson's disease (*17*−*19*). In this context, it is of interest to know whether MPTP is metabolized by P4502D enzyme(s). However, no systematic studies clearly demonstrating the participation of P4502D enzymes in MPTP *N*-demethylation have been reported so far.

The present study was conducted to determine the P450 enzymes in MPTP *N*-demethylation in rat liver microsomes as a detoxication pathway. We have studied the kinetics of MPTP *N*-demethylation in this preparation, and we report here evidence showing that P4502C11 as well as P4502D enzyme(s) is involved in the reaction.

Experimental Procedures

Materials. MPTP, PTP, and propranolol as hydrochlorides were purchased from Sigma Chemical Co. (St. Louis, MO) and the other compounds obtained as follows: β-naphthoflavone from Aldrich Chemical Co. (Milwaukee, WI); debrisoquine hemisulfate from Hoffman La-Roche Co. (Basel, Switzerland); sodium phenobarbital from Wako Pure Chemical Ind. (Osaka, Japan); SKF 525-A from Funakoshi Co. (Tokyo, Japan). Other reagents or chemicals used were of the highest quality commercially available.

Animals. Adult Wistar and Dark Agouti (DA) rats of both sexes (8 weeks old) were obtained from Takasugi Experimental Animals (Kasukabe, Japan) and SLC (Shizuoka, Japan), respectively, and allowed food and water *ad libitum*. The animals were killed by decapitation, and liver microsomes were prepared according to the method of Omura and Sato (*20*). In enzyme induction studies, male Wistar rats were given a daily dose of phenobarbital or β-naphthoflavone (each 80 mg/kg, ip, for 3 days) and were sacrificed by decapitation 24 h after the final dose, and liver microsomes were prepared as described above. In enzyme inhibition studies, the animals were given a daily dose of propranolol (100 mg/kg, po, for 5 days) and sacrificed 40 h after the final dose.

Identification of PTP by LC/MS. A reaction mixture (5 mL) contained 10 mM glucose 6-phosphate (G-6-P), 10 units of G-6-P dehydrogenase, 10 mM $MgCl_2$, 10 mg/mL microsomal protein, 50 μM MPTP, and 154 mM potassium phosphate buffer (pH 7.4). Incubation was started by adding NADPH (1 mM) and continued for 15 min. The reaction was terminated by adding ice-cold 1 N NaOH to the mixture followed by extraction with ethyl acetate (10 mL × 2). The organic layer was dried over anhydrous Na_2SO_4, and then solvent was removed with a rotary evaporator. The residue was dissolved in 100 μL of ethanol as a sample for LC/MS analysis under the conditions described below.

Assay of MPTP *N*-Demethylase Activity by HPLC. A typical reaction mixture (0.5 mL) contained 10 mM G-6-P, 1 unit of G-6-P dehydrogenase, 10 mM $MgCl_2$, 1 mg/mL microsomal protein, 5 μM MPTP, and 154 mM potassium phosphate buffer (pH 7.4). After preincubation at 37 °C for 5 min, incubation was started by adding NADPH (1 mM) and continued for 2.5 min. The reaction was terminated by adding ice-cold 1 N NaOH to the mixture and cooling at 0 °C in an ice bath. The mixture was then extracted with 5 mL of ethyl acetate by vigorous shaking, followed by centrifugation at 3000*g* for 10 min. To 4 mL of the organic layer were added an internal standard (flurazepam, 200 ng) and 0.2 mL of 0.01 N HCl. MPTP, PTP, and the internal standard in the organic layer were then back-extracted into the aqueous layer. After centrifugation, the organic layer was discarded by aspiration and the aqueous layer evaporated with a rotary evaporator. The residue was dissolved in 100 μL of an HPLC mobile phase described below. The quantities of PTP were determined by the HPLC from calibration curves prepared by adding known amounts of synthetic PTP to ice-cold reaction medium containing the same ingredients described above, and by extraction without incubation.

Reconstitution of MPTP *N*-Demethylation Using Purified P450 Enzymes. P450BTL, a member of the P4502D subfamily, was purified from liver microsomes of adult male Sprague-Dawley (SD) rats by following bunitrolol 4-hydroxylase activity as reported previously (*21*). This enzyme showed much higher activities toward bunitrolol and propranolol compared with 12 other P450 enzymes purified from rat liver in the reconstituted system, and its *N*-terminal amino acid sequence was very similar to that of P4502D2 (*21*). P450m1 (P4502C11) was purified from male rats of the same strain as described elsewhere (*22*). NADPH−P450 reductase was purified from liver microsomes of male SD rats pretreated with phenobarbital according to the method of Yasukochi and Masters (*23*). The reconstituted system contained 5 μg of dilauroylphosphatidylcholine, 10 mM G-6-P, 1 unit of G-6-P dehydrogenase, 10 mM $MgCl_2$, 0.5 unit of NADPH−P450 reductase, MPTP (2.5 nmol or 2.5 μmol), and 154 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.5 mL. The reaction at 37 °C was started by adding NADPH (1 mM), allowed to continue for 10 min, and then terminated by adding 1 N NaOH as described in the microsomal experiments.

Conditions for HPLC and LC/MS Analyses. The HPLC apparatus consisted of an LC-3A liquid chromatograph equipped with an SPD-2A UV detector and C-R6A Chromatopac data processor (Shimadzu, Kyoto, Japan). The chromatographic conditions were as follows: column, Inertsil ODS (4.6 mm i.d. × 25 cm, GL Science Co., Tokyo, Japan); column temperature, 40 °C; mobile phase, 50 mM potassium phosphate buffer (pH 7.4)/acetonitrile (55:45 v/v); flow rate, 2.0 mL/min; detection, 244 nm. LC/MS was conducted on a JEOL LX-2000 instrument under the following conditions: interface, FRIT-FAB; injection, flow injection (10 μL); matrix, 1% glycerin; collision gas; xenon.

In Vitro Inhibition Studies. SKF 525-A (a typical inhibitor of P450, 2 mM) or debrisoquine (a typical substrate of P4502D enzyme, 50−500 μM) was added to the reaction mixture containing the same ingredients as mentioned above, except that the MPTP concentrations used were 1 mM for the SKF 525-A experiment and 5 μM for the debrisoquine experiment.

Others. Protein concentrations were determined by the method of Lowry *et al*. (*24*). Enzyme kinetic parameters (K_m and V_{max}) were analyzed according to a nonlinear least-squares regression analysis based on a simplex method (*25*). Statistical significance was calculated by the Student's *t*-test.

Results

HPLC and LC/MS Analyses of the Formation of PTP from MPTP in Rat Liver Microsomes. In preliminary experiments, we confirmed that the formation of PTP increased linearly with time to 5 min and with microsomal protein concentration to 2 mg/mL. Based on these results, an incubation time of 2.5 min and a microsomal protein concentration of 1.0 mg/mL were used in subsequent experiments. Chromatogram A in Figure 2 shows a tracing of MPTP (2 nmol), PTP (2 nmol),

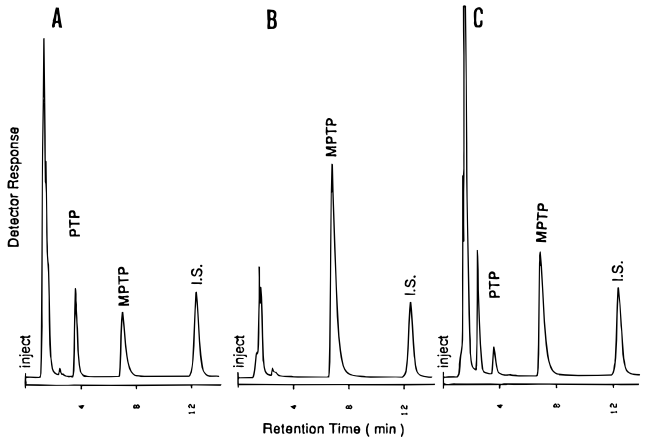

Figure 2. Typical high-performance liquid chromatograms of MPTP, PTP, and flurazepam. (A) An ethyl acetate extract of ice-cold reaction medium containing microsomes, other ingredients, and authentic MPTP (2 nmol), PTP (2 nmol), and flurazepam (200 ng); (B) an extract of reaction medium containing microsomes, MPTP (5 nmol), and flurazepam (200 ng); (C) an ethyl acetate extract of post-incubated reaction medium containing microsomes, MPTP (5 nmol), and other ingredients necessary for the monooxygenation.

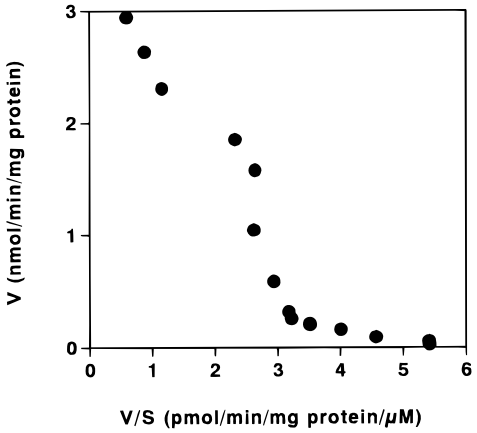

Figure 3. Typical Eadie−Hofstee plots showing MPTP *N*-demethylation in liver microsomes from male Wistar rats. The substrate concentration range was from 1.25 to 5000 μM. Kinetic parameters calculated were as follows: K_{m1} 12.9 μM, V_{max1} 0.033 nmol/(min·mg of protein) for a low K_m system; and K_{m2} 1001 μM, V_{max2} 3.44 nmol/(min·mg of protein) for a high K_m system.

and the internal standard (flurazepam, 200 ng) which were added to an ice-cold reaction mixture containing all the ingredients and extracted without incubation. The tracing of an extract of a reaction mixture which was incubated without MPTP but to which MPTP (5 nmol) and flurazepam had been added just before extraction is shown as chromatogram B. Chromatogram C is a tracing of an extract from a reaction mixture in which MPTP (5 nmol) was incubated with an NADPH-generating system. The retention times of MPTP, PTP, and the internal standard were 7.0, 3.6, and 12.4 min, respectively.

The identification of the peak with a retention time of 3.6 min as PTP was further conducted by LC/MS. The LC/MS analysis of the peak isolated from eluents of the HPLC showed that a molecular ion (*m*/*z* 160, M + H) and other fragment ions of the peak were in good agreement with those of a synthetic standard (data not shown). A peak with a retention time of 2.5 min was thought to be a mixture of two or three metabolites of MPTP, which could not be identified from their overlapped and complicated fragment ions. These results showed that MPTP *N*-demethylation could be satisfactorily assayed with the HPLC conditions shown.

Contribution of P450 to the MPTP *N*-Demethylation in Rat Liver Microsomes. To confirm that P450 is responsible for the formation of PTP from MPTP under the conditions used, we examined the effects of SKF 525-A (a typical P450 inhibitor) on the *N*-demethylase activity in liver microsomes from male Wistar rats. The activity was almost completely (over 99%) inhibited by SKF 525-A (2 mM, data not shown). Omission of NADPH, the cofactor for monooxygenation by P450, prevented the formation of PTP. These results demonstrated that MPTP *N*-demethylation was mediated by P450 enzyme in rat liver microsomes under the present conditions.

Kinetics of MPTP *N*-Demethylation. We then examined the kinetics of formation of PTP from MPTP in liver microsomes from male Wistar rats using a substrate concentration range from 1.25 to 5000 μM. The results are shown in Figure 3 as a nonlinear Eadie−Hofstee plot which indicates multiple enzyme participation. The data were analyzed in terms of a two enzyme system by nonlinear regression procedures, and the parameters indicated in the legend were obtained. The mean values of kinetic parameters obtained from three experiments with different microsomal fractions are as follows: a low K_m system, K_{m1} 10.0 ± 2.2 μM, V_{max1}, 0.048 ± 0.009 nmol/(min·mg of protein); a high K_m system, K_{m2} 1180 ± 91 μM, V_{max2} 4.80 ± 0.75 nmol/(min·mg of protein) (mean ± SE of three determinations). These results indicate that at least two P450 enzymes were involved in the MPTP *N*-demethylation in rat liver microsomes. We therefore employed two substrate concentrations (5 μM and 5 mM for the low and high K_m systems, respectively) in subsequent experiments.

Effects of Enzyme Induction on MPTP *N*-Demethylation. Figure 4 shows the effects of phenobarbital or β-naphthoflavone pretreatment of male Wistar rats on microsomal formation of PTP. Pretreatments with these inducers reduced *N*-demethylation activtiy of both the low (Figure 4A) and high K_m (Figure 4B) systems, with the drop in the activity greater in the β-naphthoflavone pretreated animals, particularly at a high substrate concentration.

Strain and Sex Differences in MPTP *N*-Demethylation. MPTP *N*-demethylase activities at a substrate concentration of 5 μM were significantly lower in DA rats than in Wistar rats (Figure 4A) and were significantly higher in male rats of both strains. At a substrate concentration of 5 mM, the sex difference became more pronounced (male ≫ female), but there was no strain difference (Figure 4B).

Effects of Enzyme Inhibition on MPTP *N*-Demethylation. In the course of our studies on the metabolic activation of propranolol, we have recognized that pretreatment of rats with propranolol causes a marked suppression in various hepatic drug oxidation activities mediated by P4502D enzyme(s) such as propranolol ring 4-, 5-, and 7-hydroxylase, debrisoquine 4-hydroxylase, and imipramine 2-hydroxylase activities (*26*). For this reason, male Wistar rats were given a daily dose of propranolol (100 mg/kg, po, for 5 consecutive days). As shown in Figure 4C, low concentration MPTP *N*-demethylase activity in liver microsomes from propranolol-pretreated rats was significantly lower than that in microsomes from rats receiving saline only and was comparable to that from male DA rats. The effect of the P4502D inhibitor, debrisoquine, on the formation of PTP was also examined. The results, shown in Figure 5,

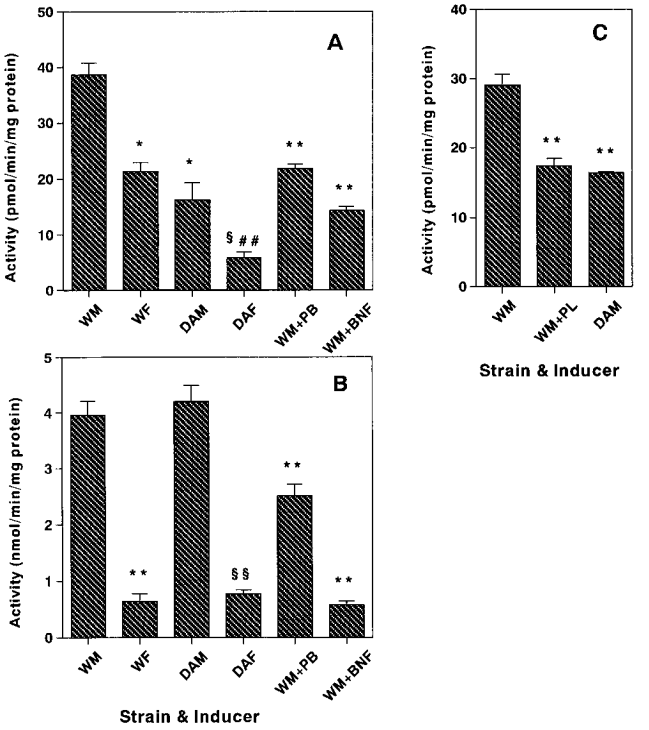

Figure 4. Effects of pretreatment of rats with P450 inducers on and strain and sex differences in MPTP *N*-demethylase activities in rat liver microsomes. For the assessment of the effects of inducers, male Wistar rats were given a daily dose of phenobarbital, β-naphthoflavone (each 80 mg/kg, ip, for 3 days), or propranolol (100 mg/kg, po, for 5 days) and were sacrificed 24 h (phenobarbital and β-naphthoflavone) or 40 h (propranolol) after the final dose. For the assessment of sex difference, liver microsomes were prepared from Wistar and DA rats of both sexes. MPTP *N*-demethylase activities were assayed using substrate concentrations of 5 μM (A and C) and 5 mM (B). Each value represents the mean ±SE of three animals. WM, DAM, and DAF mean Wistar male, Wistar female, DA male, and DA female, respectively. WM+PB, WM+BNF, and WM+PL mean phenobarbital-, β-naphthoflavone-, and propranolol-treated male Wistar rats, respectively. (*, **) Significantly different from male Wistar rats (p <0.05 and p <0.01, respectively). (§, §§) significantly different from male DA rats (p <0.05 and p <0.01, respectively). (##) Significantly different from female Wistar rats (p <0.01).

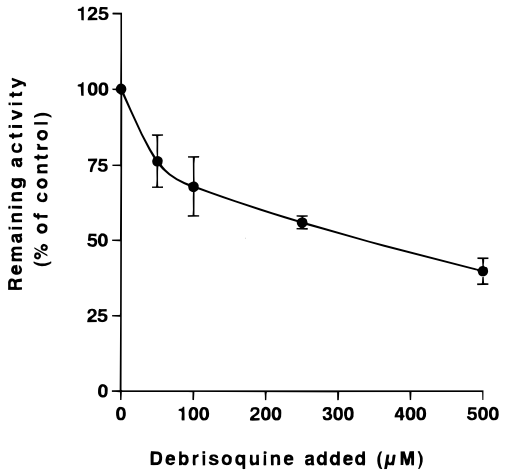

Figure 5. Effects of debrisoquine on MPTP *N*-demethylase activities in rat liver microsomes. Debrisoquine (final concentrations of 50−500 μM) was added to the incubation medium containing microsomes from male Wistar rats and other ingredients and was incubated at 37 °C for 2.5 min. Each value represents the mean ±SE of three determinations.

indicate that debrisoquine suppressed MPTP *N*-demethylase activity in a concentration-dependent manner. However, the suppression was incomplete, and about 40% of the activity remained even at the highest debrisoquine concentration of 500 μM.

Table 1. MPTP *N*-Demethylase Activity of Purified P450 Enzymes in the Reconstituted System[a]

enzymes	MPTP *N*-demethylase act. (nmol/(min·nmol of P450)) at a substrate concn of: 5 μM	5 mM
P450BTL	0.19	0.23
P450m1	0.57	101.8

[a] The reconstituted system (0.5 mL) contained purified P450 (50 pmol), NADPH−P450 reductase (0.5 unit), sodium cholate (0.1 mg), dilauroylphosphatidylcholine (5 μg), NADPH (0.5 μmol), MPTP (2.5 nmol or 2.5 μmol), the NADPH-generating system, and potassium phoshate buffer (pH 7.4, 77 μmol). Each value represents the mean of two determinations.

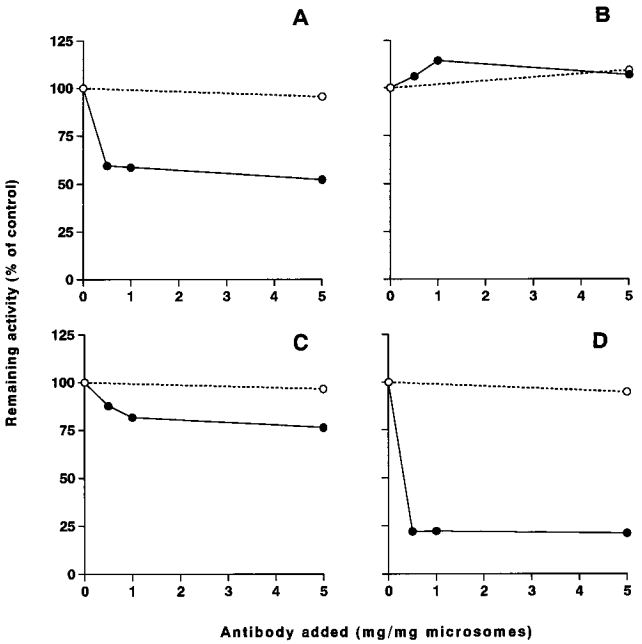

Figure 6. Effects of antibodies against P450BTL and P450m1 on MPTP *N*-demethylase activities in rat liver microsomes. Polyclonal antibodies against P450BTL or P450m1 were preincubated with liver microsomes from male Wistar rats at 25 °C for 30 min. MPTP and an NADPH-generating system were then added, and incubation at 37 °C was performed. Closed circles, anti-P450 IgG; open circles, preimmune IgG. Left panels (A and C), low substrate concentration (5 μM); right panels (B and D), high substrate concentration (5 mM); upper panels (A and B), anti-P450BTL; lower panels (C and D), anti-P450m1. Each point was the mean of duplicated experiments.

Reconstitution of MPTP *N*-Demethylation. MPTP *N*-demethylase activity was then reconstituted, using purified P450 enzymes (P450BTL and P450m1), NADPH−P450 reductase, dilauroylphosphatidylcholine, and an NADPH-generating system (Table 1). P450m1 showed PTP-forming activity higher than that of P450BTL at both substrate concentrations of 5 μM and 5 mM. At 5 mM, the activity of P450m1 was 101.8 nmol/(min·nmol of P450), which was 446-fold higher than that of P450BTL.

Effects of Antibodies against P450 Enzymes on Microsomal MPTP *N*-Demethylation. Liver microsomes from male Wistar rats were preincubated with antibodies raised against P450BTL or P450m1, and microsomal MPTP *N*-demethylase activity was assayed using the two substrate concentrations. As shown in Figure 6, antibodies against P450BTL suppressed the formation of PTP from MPTP at a low substrate concentration (5 μM) by 48% at an antibody to microsomal protein ratio of 5 (Figure 6A), whereas antibodies against

P450m1 suppressed the activity only by 22% even at the highest protein ratio (Figure 6C). When the high substrate concentration (5 mM) was employed, on the other hand, the antibodies against P450BTL had no effect on the activity (Figure 6B), whereas the antibodies against P450m1 reduced MPTP *N*-demethylation activity to 22% of control at an antibody to microsome protein ratio of 1.0 (Figure 6D).

Discussion

In the present study, MPTP *N*-demethylation in liver microsomes from male Wistar rats was found to be biphasic, *i.e.*, consistent with two enzyme systems. This means at least two P450 enzymes are involved in the formation of PTP from MPTP in male rat liver microsomes. Chiba *et al*. (*8*) reported the kinetics of PTP from MPTP in mouse liver microsomes was monophasic, with a K_m value of 814 ± 71 μM and a maximum velocity of 4.42 ± 0.28 nmol/(min·mg of protein). These parameter values are similar to those for the high K_m system (K_{m2}, 1180 ± 91 μM; V_{max2}, 4.80 ± 0.75 nmol/(min·mg of protein)) in rat liver microsomes in our study.

They also observed that the pretreatment of mice with phenobarbital or 3-methylcholanthrene increased the clearance (*8*), suggesting the involvement of P450 enzymes belonging to the P4502B or -1A subfamilies in mouse liver microsomal MPTP *N*-demethylation. Contrary to the results in the mouse (*8*), however, pretreatments of rats with P450 inducers such as phenobarbital and β-naphthoflavone markedly decreased the microsome mediated PTP formation, so it is unlikely that P450 enzymes belonging to the P4501A or -2B subfamily are involved in MPTP *N*-demethylation by this species.

MPTP *N*-demethylase activity was significantly lower in the P4502D deficient DA rat (*27*) than in Wistar rats, particularly at a low substrate concentration (5 μM), indicating that P4502D enzyme(s) were involved in rat liver microsomal MPTP *N*-demethylation. Previous studies (*26*) had shown that propranolol selectivity inhibits P4502D enzymes. We thus examined MPTP *N*-demethylase activities in liver microsomes from rats pretreated with propranolol and found that the activity of male Wistar rats receiving the propranolol pretreatment was decreased to the level of native male DA rats, whose activity was significantly lower than that of native male Wistar rats. These results indicated that P4502D enzyme(s) are involved in MPTP *N*-demethylation in a low substrate concentration range.

We observed a marked sex difference (male > female) in MPTP *N*-demethylase activities in liver microsomes from Wistar and DA rats, especially at a high substrate concentration. It should be noted that there was no strain difference between Wistar and DA rats in MPTP *N*-demethylase activities at a MPTP concentration of 5 mM (Figure 4B). These results indicate that male-specific P450 enzyme(s) are the major contributors to PTP formation in male rat liver microsomes as the major P450 at higher substrate concentration range.

To confirm possible involvement of P4502D and male-specific P450 enzyme(s) in rat liver microsome mediated MPTP *N*-demethylation, we reconstituted this enzyme activity using P450BTL and P450m1, which corresponds to male-specific P4502C11 (*22*, *28*). The activities of P450m1 were higher than those of P450BTL in substrate concentrations of 5 μM and 5 mM. To our surprise, the activity of the male-specific enzyme was very high in the substrate concentration of 5 mM, which was over 100 nmol/(min·nmol of P450). Moreover, the immunochemical studies demonstrated that antibodies against P450BTL suppressed half of the activity at the low MPTP concentration, whereas antibodies against P450m1 suppressed most of the activity at the high MPTP concentration. These results indicate that major P450 enzymes catalyzing MPTP *N*-demethylation are P4502D enzyme(s) as a low K_m enzyme in a lower substrate concentration range and P4502C11 as a high K_m enzyme in a higher substrate concentration range in rat liver microsomes.

The present study showed that MPTP *N*-demethylase activity was lower in DA rats than in Wistar at a lower substrate concentration. Furthermore, a male-specific P4502C11 is supposed to contribute to PTP formation at higher substrate concentrations. These results and findings suggest that lower MPTP *N*-demethylase activity makes female DA rats more sensitive to neurotoxicity of MPTP than male rats of other strains. In fact, Jimenez-Jimenez *et al*., (*29*) reported that female DA rats are more susceptible to acute neurotoxic effects of MPTP than other strain rats such as Fischer-344 or Wistar. However, there have been no reliable reports showing a clear sex difference in MPTP neurotoxicity in any animal species including the rat.

We reported previously that 1,2,3,4-tetrahydroisoquinoline, another compound that induces a Parkinsonism-like state in experimental animals, was converted to a nontoxic 4-hydroxylated metabolite mainly by P4502D enzyme(s) in the rat (*30*). The results of the present study show that metabolism of MPTP by P4502D enzyme(s) and P4502C11 is a detoxication pathway in male rat liver. It is known that mammalian brains contain various P450 enzymes including the P4502D subfamily (*31*, *32*). These results and findings do not directly support the possible correlation between the debrisoquine hydroxylase deficient poor metabolizer genotype and Parkinsonism (*17*−*19*, *33*), but instead suggest that P4502D enzymes are involved in the detoxication of these neurotoxic substances in mammalian brains as well as in livers. It is tempting to think that, in the brain, some interaction between P4502D enzyme(s) and neurotoxins such as MPTP or 1,2,3,4-tetrahydroisoquinoline in the situation of enzyme−substrate or enzyme−inhibitor would be one of causes correlating P4502D polymorphism with Parkinson's disease, but supporting data are still insufficient. Further systematic studies focusing on this point are needed.

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