METABOLISM OF 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP) IN PERFUSED RAT LIVER: INVOLVEMENT OF HEPATIC ALDEHYDE OXIDASE AS A DETOXIFICATION ENZYME

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ABSTRACT:
To elucidate the toxicological relevance of hepatic aldehyde oxidase (AO) as a detoxification enzyme of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), we studied the metabolism and the hepatotoxicity of MPTP in intact rat livers exhibiting different AO activities by using a recirculating perfusion method. In the perfusate during a 90-min recirculation of 1 mM MPTP, the perfused liver from Jcl:Wistar rat, a strain showing high AO activity, generated almost equal amounts of 1-methyl-4-phenylpyridinium species (MPP⁺) and 1-methyl-4-phenyl-5,6-dihydro-2-pyridone (MPTP lactam) as major metabolites, together with 4-phenyl-1,2,3,6-tetrahydropyridine, 1-methyl-4-phenyl-2-pyridone (MP 2-pyridone) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine N-oxide. However, a marked decrease of MPTP lactam as well as MP 2-pyridone and a concomitant increase of MPP⁺ were caused by coinfusion of 2-hydroxy-2-pyrimidinone (2-OH PM), a competitive inhibitor of AO, into Jcl:Wistar rat liver. A quite similar metabolic profile was obtained on perfusion of AO-deficient WKA/Sea rat liver. Rather large amounts of MPP⁺ were retained in the liver in all cases, but especially in Jcl:Wistar rat in the presence of 2-OH PM. Lactate dehydrogenase leakage into the perfusate from rat liver perfused with 1 mM MPTP was greater in the strain with lower AO activity, WKA/Sea, than in that with higher AO activity, Jcl:Wistar. Furthermore, inhibition of AO in Jcl:Wistar rat in the presence of 2-OH PM caused an enhancement of lactate dehydrogenase leakage. These results suggest that hepatic AO is a key detoxification enzyme for MPTP.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a potent neurotoxin that destroys dopaminergic neurons of the substantia nigra and thereby causes typical parkinsonism in humans and other primates (Burns et al., 1983; Langston et al., 1983, 1984). Several groups of investigators have demonstrated that the ultimate toxic metabolite of MPTP is 1-methyl-4-phenylpyridinium species (MPP⁺) generated by either enzymatic or nonenzymatic oxidation of 1-methyl-4-phenyl-2,3-dihydropyridine species (MPPDP⁺), which is a primary oxidation product of MPTP by mitochondrial monoamine oxidase-B (MAO-B) in brain tissue (Chiba et al., 1988). MPP⁺ incorporated into dopaminergic neurons blocks mitochondrial electron transport by inhibition of complex I and results in ATP depletion, leading to cell death (Nicklas et al., 1987; Trevor et al., 1987; Singer and Ramsay, 1990).

In the liver, a major site of metabolism of xenobiotics, MPTP is also metabolically activated to MPP⁺ via MPDP⁺ by MAO-B and causes hepatocyte toxicity (Di Monte et al., 1987; Smith et al., 1987). MPTP is metabolized to 4-phenyl-1,2,3,6-tetrahydropyridine (PTP) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine N-oxide (MPTP N-oxide) by liver microsomal cytochrome P-450 (P450; Weissman et al., 1985; Coleman et al., 1996) and flavin-containing monoxygenase (FMO; Cashman and Ziegler, 1986; Di Monte et al., 1988), respectively. These metabolic reactions are considered to be inactivation pathways of MPTP (Smith et al., 1987; Chiba et al., 1988). Two pyridones, 1-methyl-4-phenyl-5,6-dihydro-2-pyridone (MPTP lactam) and 1-methyl-4-phenyl-2-pyridone (MP 2-pyridone), have also been identified as metabolites either in vivo (Arora et al., 1988) or in vitro (Baker et al., 1984; Wu et al., 1988) in rodents. Recently, we demonstrated that in rodents, hepatic aldehyde oxidase is an important detoxification enzyme to scavenge the ultimate toxic MPP⁺ at least at the hepatic level.

There are marked strain differences of hepatic AO activity in rats: Jcl:Wistar strain is classified as having higher AO activity, whereas WKA/Sea is an AO-deficient strain (Sugihara et al., 1995). Therefore, to elucidate the toxicological relevance of hepatic AO as a detoxification enzyme of systemically administered MPTP, we investigated the metabolism of MPTP in intact liver of rat strains with different AO activities by means of a recirculating perfusion method and assessed
the hepatotoxicity by measuring lactate dehydrogenase (LDH) leakage into the perfusate.

Materials and Methods

Chemicals. The sources of materials used were as follows: MPTP hydrochloride and MPP⁺ iodide were obtained from Research Biochemicals International (Natick, MA); PTP and 2-hydroxypyrimidine (2-OH PM) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); and 4-phenylpyridine (4-PP) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). MPTP lactam and MP 2-pyridone were synthesized by the method of Wu et al. (1988) and MPTP N-oxide was synthesized according to the method of Weissman et al. (1985). Other chemicals were of the highest quality commercially available.

Liver Perfusion. Male Jcl:Wistar and WKA/Sea rats at 5 to 6 weeks of age were purchased from CLEA Japan, Inc. (Tokyo, Japan) and Seac Yoshitomi, Ltd. (Fukuoka, Japan), respectively. After acclimation for about 2 weeks, these animals were anesthetized with pentobarbital for surgical operation. The livers (about 10–15 g) of nonfasting rats were perfused in situ by a recirculating method for 90 min at 32°C. The perfusion fluid was Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 5 mM glucose, saturated with 95% O₂ and 5% CO₂ (Yoshihara and Tatsumi, 1995). The influent perfusate (200 ml), which initially contained 1 mM MPTP with or without 5 mM 2-OH PM, was pumped via a cannula into the portal vein at a flow rate of approximately 3 ml/min/g liver, and the effluent perfusate was passed into a perfusate reservoir through the thoracic vena cava. Aliquots (3.5-ml) of the recirculated perfusate were withdrawn from the reservoir for the measurements of the metabolites and LDH activity at the designated time points. After 90 min, the liver was isolated from the carcass and homogenized with 4 volumes of 25 mM potassium phosphate buffer (pH 7.4) to determine the metabolites retained.

Metabolite Measurement. To determine the metabolites in the perfusate, a 1-ml sample was vortexed with 2 ml of ice-cold acetonitrile containing 50 nmol of 4-PP as an internal standard. For the determination of the metabolites retained in the perfused liver, 0.2 ml of the whole homogenate was diluted with 0.8 ml of 25 mM potassium phosphate buffer (pH 7.4), and then vortexed with 2 ml of ice-cold acetonitrile containing the same amount of 4-PP as above. The mixture was allowed to stand for 20 min on ice, and then was centrifuged at 2500 rpm for 10 min. Ten microliters of the resultant supernatant was analyzed by HPLC using a reversed-phase column Supelcosil RKb-100 (4.6 × 150 mm, 5 µm; Supelco, Inc., Bellefonte, PA) as described previously (Yoshihara and Ohta, 1998). The mobile phase was a mixture of 25% acetonitrile and 75% 25 mM potassium phosphate (pH 7.4) at a flow rate of 1 ml/min, and monitoring was performed at 265 nm.

Fig. 1. HPLC profiles of MPTP metabolites in the perfusate of Jcl:Wistar rat liver in the absence and presence of 2-OH PM.

Jcl:Wistar rat livers were perfused with 1 mM MPTP in the absence (A) and presence (B) of 5 mM 2-OH PM by a recirculation method as described under Materials and Methods. An aliquot of the perfusate after a 90-min recirculation was analyzed by HPLC. Peaks on the chromatograms are as follows: 1, MPTP N-oxide; 2, MPP⁺; 3, PTP; 4, MP 2-pyridone; 5, MPTP lactam; 6, MPTP; 7, 4-PP (internal standard); 8, Unknown metabolite.
LDH Leakage. The activity of LDH in 0.5 ml of the perfusate was monitored in terms of the decrease of absorbance at 340 nm due to NADH using sodium pyruvate as a substrate (Wroblewski and La Due, 1955).

Results

Metabolism of MPTP in Perfused Rat Liver. Figure 1 shows HPLC chromatograms of MPTP metabolites in the perfusate after a 90-min recirculation of 1 mM MPTP in Jcl:Wistar rat liver in the absence or presence of 5 mM 2-OH PM, which is an efficient substrate of AO (Yoshihara and Tatsumi, 1985) and was used as a competitive inhibitor in this experiment. MPTP lactam and an unknown metabolite with the retention time of 4.15 min, as well as MPP\(^+\) and MPTP N-oxide, were major metabolites in the intact liver of the high AO activity strain Jcl:Wistar in the absence of 2-OH PM (Fig. 1A). PTP and MP 2-pyridone were minor metabolites. In contrast, when 2-OH PM was infused, both the lactam and the unknown metabolite were markedly decreased and MPP\(^+\) was concomitantly increased (Fig. 1B). Interestingly, the HPLC profile obtained after liver perfusion of the AO-deficient strain WKA/Sea was quite similar to that obtained in the case of Jcl:Wistar liver perfused in the presence of 2-OH PM (Fig. 2). Time-dependent formation of MPTP lactam in the perfusate of the two strains is shown in Fig. 3. Large amounts of the lactam were found in the perfusate of the high AO strain Jcl:Wistar up to 30 min. However, in the perfusate of WKA/Sea liver and that of Jcl:Wistar liver in the presence of 2-OH PM, only small amounts of the lactam were detected throughout the recirculation. Figure 4 shows the time-dependent formation of toxic MPP\(^+\) in the perfusate of the two strains. Both strains gave comparable amounts of MPP\(^+\) in the perfusate, whereas coinfusion of 2-OH PM in the Jcl:Wistar liver caused a higher accumulation of MPP\(^+\).

The findings on MPTP metabolism in the perfused rat livers are summarized in Table 1. The major metabolites in the perfusate of Jcl:Wistar rat liver after a 90-min perfusion were MPP\(^+\), MPTP lactam, and MPTP N-oxide, followed by PTP and MP 2-pyridone. A marked decrease of the lactam and MP 2-pyridone was caused by coinfusion of 2-OH PM in the liver of this strain, with a compensatory increase of MPP\(^+\). In addition, MPTP N-oxide was also increased, whereas PTP was somewhat decreased in the presence of 2-OH PM. In the perfusate of WKA/Sea rat liver, the level of MPP\(^+\) was similar to that in Jcl:Wistar rat liver, but the amounts of MPTP lactam and MP 2-pyridone were similar to those in Jcl:Wistar rat liver perfused in the
presence of 2-OH PM. Marked accumulation of MPP⁺ was observed in the perfused livers, whereas other metabolites including the lactam were found at similar levels to those in the perfusates.

The amounts of MPTP recovered at the end of perfusion were almost similar (about 75 µmol) in Jcl:Wistar rat liver in the absence or presence of 2-OH PM, but somewhat greater (about 100 µmol) in WKA/Sea rat liver than those in Jcl:Wistar (data not shown). In all cases, total recoveries, which include six metabolites identified and unchanged MPTP, but not the unknown metabolite, were about 85% (data not shown).

**LDH Leakage into the Perfusate.** The activities of LDH leaked into the perfusates during recirculating perfusion are shown in Fig. 5. Almost no leakage of LDH was observed in the control perfusion of the livers of both strains. However, a gradual increase of LDH leakage was observed in the perfusate of Jcl:Wistar rat liver during recirculating perfusion with 1 mM MPTP. This LDH leakage was enhanced by coinfusion of 2-OH PM, which alone caused almost no increase of the leakage (data not shown). In the perfusate of WKA/Sea rat liver perfused with MPTP, high levels of leakage of LDH were observed.

**TABLE 1**

<table>
<thead>
<tr>
<th>Chemicals Infused</th>
<th>Strain of Rat</th>
<th>MPTP Lactam</th>
<th>MP 2-Pyridone</th>
<th>PTP</th>
<th>MPTP N-Oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPTP</td>
<td>Jcl:Wistar</td>
<td>0.90</td>
<td>0.11</td>
<td>0.32</td>
<td>0.68</td>
</tr>
<tr>
<td>MPTP + 2-OH PM</td>
<td>Jcl:Wistar</td>
<td>1.50</td>
<td>0.11</td>
<td>0.39</td>
<td>0.68</td>
</tr>
<tr>
<td>MPTP</td>
<td>WKA/Sea</td>
<td>0.92</td>
<td>0.05</td>
<td>0.23</td>
<td>0.94</td>
</tr>
</tbody>
</table>

**Discussion**

Systemically administered MPTP can reach the substantia nigra of the brain by passing through the blood-brain barrier and is oxidized to MPDP⁺ by mitochondrial MAO-B in astrocytes (Ramson et al., 1987; Di Monte et al., 1991). This unstable intermediate is also converted to an active metabolite, MPP⁺, by either enzymatic or nonenzymatic oxidation (Castagnoli et al., 1985). Therefore, it seems very likely that the neurotoxicity of systemically administered MPTP is strongly dependent on the amount of MPTP that escapes hepatic clearance and reaches the substantia nigra.

AO, a cytosolic molybdoflavoenzyme, catalyzes the oxidation of a wide range of endogenous and exogenous N-heterocycles and aldehydes with a different substrate specificity from that of microsomal monoxygenases such as P450 and FMO (Beedham, 1985; Yoshihara and Tatsumi, 1997). In previous studies using a cell-free system, we have demonstrated that hepatic AO in rodents can prevent the formation of MPP⁺, an ultimate toxic metabolite of MPTP, by catalyzing conversion of MPDP⁺ to MPTP lactam (Yoshihara and Ohta, 1998). From a toxicological viewpoint, it is clearly important to elucidate the involvement of AO in MPTP metabolism in the intact rat liver in relation to the hepatotoxicity caused by MPTP. In rats, hepatic AO activity is highly strain-dependent. Jcl:Wistar rats exhibit high activity, whereas WKA/Sea rats show almost negligible activity (Sugihara et al., 1995). We therefore used these two strains for a liver perfusion study to evaluate the role of AO in MPTP metabolism in intact liver and to establish how it affects hepatotoxicity.

In the perfused rat livers, at least seven or eight metabolites, including unknown metabolites, were detected by HPLC (Figs. 1 and 2). MPP⁺, which is primarily generated by MAO-B and predominantly retained in hepatocytes (Di Monte et al., 1988), was a major metabolite in both strains. MPTP lactam was also a major metabolite in the perfused liver from Jcl:Wistar rat with high AO activity, whereas its formation was negligible in AO-deficient WKA/Sea rat. Furthermore, coinfusion of 2-OH PM as a competitive inhibitor of AO in Jcl:Wistar rat liver resulted in a dramatic decrease in formation of the lactam and MP 2-pyridone. These findings are consistent with the results of cell-free experiments, which demonstrated the involvement of AO in producing the lactam (Yoshihara and Ohta, 1998). MP 2-pyridone is a secondary metabolite of the lactam (Wu et al., 1988). The unknown metabolite with a retention time of 4.15 min appears to be another AO-dependent metabolite because its formation was also strongly inhibited by 2-OH PM. A preliminary experiment suggested that this unknown metabolite is a secondary metabolite of MPTP lactam produced by an NADPH-dependent microsomal enzyme system such as P450 (our unpublished observations). In relation to this observation together with that by Wu et al. (1988), MPTP lactam...
formation reached a plateau at 30 min after perfusion (Fig. 3) whereas concentrations of both MP 2-pyridone and the unknown metabolite in the perfusate continued to ascend during a 90-min perfusion (data not shown), indicating that the lactam formed is also metabolized to these secondary metabolites. These results indicate that the lactam formation reaction is a predominant metabolic route of MPTP in Jcl:Wistar rat liver in the absence of 2-OH PM (Fig. 6). In Jcl:Wistar rat liver coinfused with 2-OH PM, the lactam formation was suppressed, and a compensatory increase of MPP+ formation was observed. This result is concordant with the fact that MPDP+ is a common intermediate of both MPP+ and MPTP lactam (Yoshihara and Ohta, 1998). Most of the MPP+ formed was retained in the liver rather than in the perfusate (Table 1).

By coinfusion of 2-OH PM in Jcl:Wistar rat liver, the formations of two monoxygenase-dependent metabolites such as MPTP N-oxide and PTP were also affected as follows: MPTP N-oxide formation was increased by 3-fold, but PTP formation was somewhat decreased. These effects by 2-OH PM may be due to neither an enhancement of FMO activity nor to an inhibition of P450, because almost no effect was observed on both activities by the addition of 2-OH PM in the cell-free systems using rat liver microsomes and S9 as the enzyme source (our unpublished observations). Smith et al. (1987) have proposed that the P450-dependent monoxygenase system might be an important detoxification pathway in hepatocytes, because MPTP cytotoxicity was potentiated by pretreatment of hepatocytes with P450 inhibitors such as SKF 525A and metyrapone. It is noteworthy, however, that SKF 525A is also a potent inhibitor of AO (Yoshihara and Tatsumi, 1985), whereas metyrapone is a substrate of AO (Usansky and Damani, 1983). These facts suggest that the enhancement of the hepatotoxicity observed with SKF 525A and metyrapone might be related to their effects on AO. Another interesting result obtained in this experiment is that LDH leakage in WKA/Sea rat liver at later time points of perfusion is greater than Jcl:Wistar rat liver in the absence of 2-OH PM (Fig. 5), whereas both strains give comparable amounts of MPP+ either in the perfusate (Fig. 4) or liver tissue (Table 1). This may indicate that there is a strain difference in the susceptibility in Jcl:Wistar and WKA/Sea rats toward MPTP toxicity.

In conclusion, hepatic AO can efficiently catalyze the metabolic conversion of MPDP+ to MPTP lactam in the intact rat liver and reduce the formation of the ultimate toxin MPP+. This in turn might reduce the hepatotoxicity. Therefore, hepatic AO may be the key detoxification enzyme in the hepatic metabolism of the neurotoxin MPTP.

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