ROLE OF 1-METHYL-4-PHENYLPIRIDINIUM ION FORMATION AND ACCUMULATION IN 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE TOXICITY TO ISOLATED HEPATOCYTES

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SUMMARY

The parkinsonian-inducing compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is converted by isolated hepatocytes to its primary metabolite, the 1-methyl-4-phenyl-2,3-dihydropyridinium ion (MPDP⁺), and to its fully oxidized derivative, 1-methyl-4-phenylpyridinium ion (MPP⁺). Only the latter, however, accumulates in the cells. Incubation of hepatocytes in the presence of MPDP⁺ also results in the selective intracellular accumulation of MPP⁺. Conversion to MPP⁺ is more rapid and extensive after exposure to MPDP⁺, than with MPTP and the former is also more toxic. Addition of MPP⁺ itself is toxic to hepatocytes but only after a long lag period, which presumably reflects its limited access to the cell and its relatively slow intracellular accumulation. As previously shown with MPTP and MPP⁺, the cytotoxicity of MPDP⁺ is dose-dependent and is consistently preceded by complete depletion of intracellular ATP. Similar to MPP⁺ but not MPTP, MPDP⁺ causes a comparable rate and extent of cytotoxicity and ATP loss in hepatocytes pretreated with the monoamine oxidase inhibitor pargyline. Pargyline blocks hepatocyte biotransformation of MPTP to MPP⁺, but it has no significant effect on MPP⁺ accumulation after exposure to either MPDP⁺ or MPP⁺. It is concluded that MPTP is toxic to hepatocytes via its monoamine oxidase-dependent metabolism and that MPP⁺ is likely to be the ultimate toxic metabolite which accumulates in the cell, causing ATP depletion and eventual cell death.

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Abbreviations: MAO, monoamine oxidase, MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPDP⁺, 1-methyl-4-phenyl-2,3-dihydropyridinium ion, MPP⁺, 1-methyl-4-phenylpyridinium ion, 1,2-MPDP, 1-methyl-4-phenyl-1,2-dihydropyridine, PTP, 4-phenyl-1,2,3,6-tetrahydropyridine

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INTRODUCTION

The cyclic tertiary allylamine MPTP destroys the dopaminergic neurons of the substantia nigra and, as a consequence, produces a clinical syndrome in humans similar to that ascribed to idiopathic Parkinson's disease [1,2]. This injury to the nigrostriatal system is dependent on the MAO B catalyzed biotransformation of the parent drug which forms initially MPDP\(^+\) and subsequently the fully aromatized 1-methyl-4-phenylpyridinium species MPP\(^+\) [3] (Fig. 1). Several lines of evidence point to MPP\(^+\) as the agent which ultimately mediates the neurotoxicity of MPTP. For example, MPP\(^+\) binds to neuromelanin [4] and accumulates in the nigral cell bodies of the susceptible primate species but not in resistant rodent species [5]. Furthermore, MPP\(^+\) is concentrated by the dopamine uptake system present in the membranes of striatal synaptosomes [6]. Finally, direct injections of MPP\(^+\) into the substantia nigra of rats causes the loss of striatal dopamine stores [7].

The mechanism by which MPP\(^+\) expresses its toxicity remains unclear. Its structural similarity to the 1,1'-dimethyl-4,4'-bipyridinium species paraquat (PQ\(^{2+}\)), a potent herbicide and pneumotoxicant [8], has led some investigators [9] to speculate that the toxicity of MPTP may be mediated by 'oxidative stress,' a process which reportedly is responsible for the toxicity of PQ\(^{2+}\). Studies with subcellular fractions derived from liver and brain tissues have established that MPP\(^+\) blocks mitochondrial respiration by inhibiting ADP-stimulated oxidation of NAD-linked substrates [10]. Additional studies have shown that both intact liver [11] and brain [12] mitochondria concentrate MPP\(^+\) in an energy dependent process. The detailed molecular events which lead to cell death, however, have not been identified.

Although a number of the toxic effects of MPP\(^+\) have been documented, relatively little is known about the possible cytotoxic effects of MPDP\(^+\), the primary MAO B derived metabolite of MPTP. MPDP\(^+\) has been reported to inhibit the uptake of dopamine by striatal synaptosomes and to deplete striatal dopamine stores following intranigral injections [13]. MPDP\(^+\) also has been found to inhibit mitochondrial respiration more efficiently than MPP\(^+\) (T.P. Singer, pers. comm.). This 2,3-dihydropyridinium species is, however, relatively unstable and it undergoes a bimolecular disproportionation reaction to yield MPTP and MPP\(^+\) [14]. It is also rapidly oxidized to MPP\(^+\) by synthetic dopamine melanin and human neuromelanin in a process which involves direct transfer of electrons to the polymeric pigment [15]. Consequently, an assessment of the possible contributions that MPDP\(^+\) may make to the toxic effects produced by MPTP will require quantitative estimations of this dihydropyridinium compound as well as MPP\(^+\) and MPTP in the system under study.

The nigrostriatal toxicity of MPP\(^+\) and MPDP\(^+\) cannot be studied readily in
vivo because of their limited access to the central nervous system. Isolated hepatocytes have served as a useful in vitro model for general studies of drug metabolism and for delineating cytotoxic mechanisms [16] including those involving oxidative stress caused by PQ^{2+} [17]. Previous work in our laboratory has shown that both MPTP and MPP^{+} are toxic to hepatocytes [18,19]. Moreover, the usual criteria attending cytotoxicity due to oxidative stress in this system are not expressed in the presence of MPP^{+} [18]. The toxicity of both compounds is, however, preceded by a dramatic depletion of ATP [19], an observation consistent with the reports that MPP^{+} inhibits mitochondrial respiration.

The present contribution extends these observations to MPDP^{+}. We also provide for the first time quantitative information of the biotransformation and disposition of MPTP, MPDP^{+} and MPP^{+} in a cellular system and correlate this with the toxic effects observed.

MATERIALS AND METHODS

**Materials**

MPTP, MPDP^{+} and MPP^{+} were obtained from Research Biochemicals (Wayland, MA) and \[^{3}H\]MPP^{+} (labelled on the N-methyl group, 85 Ci/mmol) from New England Nuclear, Boston, MA. All other chemicals were of the highest purity available and were purchased from Sigma Chemical Co., St. Louis, MO.

**Hepatocyte isolation and incubation**

Hepatocytes were isolated from male Sprague–Dawley rats (200–250 g, given food and water ad libitum) by collagenase perfusion of the liver [20]. They were incubated (10^6 cells/ml) in Krebs-Henseleit buffer supplemented with 12.6 mM Hepes (pH 7.4), under a 95% O_2:5% CO_2 atmosphere in rotating round bottom flasks. Cell viability was assessed as the percentage of hepatocytes which excluded a 0.16% solution of Trypan blue dye. The percentage of viable cells was always greater than 90% at the beginning of the experiments. When inhibition of MAO was desired the cells were preincubated for 10 min with 10^{-6} M pargyline prior to addition of the pyridine compounds.

**ATP content of hepatocytes**

Samples of the hepatocyte incubation mixtures (2 ml) were taken at specific intervals and centrifuged at low speed for 60 s. The supernatant fractions were separated and the cell pellets resuspended in 1 ml of ice-cold water. Perchloric acid (3 N) was added (1:5, v/v) to precipitate proteins. Intracellular ATP was measured spectrophotometrically (340 nm) from the clear supernatant as NADPH formation in the presence of NADP^{+}, glucose, hexokinase and glucose-6-phosphate dehydrogenase [21]. External and internal standards were used for exact quantitative estimations. Comparable results were obtained when ATP was measured by a chemiluminescence procedure [22].
Analysis of MPTP and metabolites

To measure intracellular concentrations of MPTP, MPDP⁺ and MPP⁺, incubation mixtures were centrifuged (1500 g for 90 s) through a suspension of Percoll in Ca²⁺ and Mg²⁺-free Hank's solution (final density, 1.06 g/ml). The 'media' fraction on top of the Percoll suspension and the pellet (intact cells) fraction were immediately frozen by immersion in dry ice/ethanol and were stored at –70°C. In some experiments [³H]MPP⁺ (6 × 10⁶ dpm, 1.5 mM) was added to the initial incubation mixture. Cell pellets were obtained as described above and were washed with Krebs-Henseleit buffer in the absence or presence of unlabeled MPP⁺ (1.5 mM). The radioactivity associated with hepatocytes was assayed by liquid scintillation counting after sonication.

For quantitative estimations, the frozen hepatocyte pellets were thawed and vortexed with 0.5 ml of cold 5% trichloroacetic acid containing 1.25 µg of 4-phenylpyridine as internal standard. The resulting mixture was allowed to stand for 1 hour at 0°C and centrifuged at 13 000 g for 5 min. The supernatant was passed through a 0.2-µm nylon filter and a 50-µl aliquot of the filtrate was analyzed by HPLC (see below). The media fraction (0.2 ml) obtained from the Percoll separation was thawed and mixed with 0.2 ml of cold 5% trichloroacetic acid and the resulting mixture was treated as described above for the pellet fraction. In addition to MPTP, MPDP⁺ and MPP⁺, these fractions were analyzed for 4-phenyl-1,2,3,6-tetrahydropyridine (PTP), the cytochrome P-450 catalyzed N-demethylation metabolite of MPTP [23], and MPTP-N-oxide, the flavin monoxygenase generated metabolite of MPTP [24] (Fig. 1). Cell pellet protein values were measured by the method of Lowry et al. [25].

![Chemical structures](image)

Fig 1 Structures of MPTP and its principal metabolites
HPLC analyses were performed with a Beckman model 114 M chromatograph equipped with an Altex Ultrasil SCX column (10 µm, 4.6 mm × 2.5 cm). The mobile phase was delivered at a flow rate of 1.5 ml/min and consisted of 90% of a mixture of 0.1 M acetic acid and 0.075 M triethylamine hydrogen chloride (adjusted to pH 2.3 with formic acid) and 10% acetonitrile. HPLC effluents were analyzed with the aid of a Hewlett-Packard model 1040A diode-array detector. The wavelengths monitored were 345 and 295 nm for MPDP⁺ and MPP⁺, respectively, and 244 nm for MPTP, MPTP-N-oxide and PTP.

RESULTS

**MPDP⁺ toxicity in isolated hepatocytes**

In Table I we compare the effects of MPTP, MPDP⁺ and MPP⁺ (all at 1.5 mM) on the survival time of freshly isolated hepatocytes. All three compounds caused 100% cell death at time points where approx. 90% of the untreated cells remained viable. However, the loss of viability was clearly most rapid after MPDP⁺ addition and significantly slower in the presence of MPP⁺. MPDP⁺, is therefore more toxic to isolated hepatocytes than both the other MAO-generated metabolite MPP⁺ and the parent compound MPTP. Similar to MPTP and MPP⁺ [19], MPDP⁺ toxicity was dose-dependent, in that comparable rates of cell death occurred at earlier time points as its concentration was increased (Fig. 2A). Furthermore, MPDP⁺ caused a dramatic dose-dependent depletion of intracellular ATP, which, as in cells exposed to MPTP or MPP⁺ [19], preceeded the onset of hepatocyte toxicity (Fig. 2B). Exposure to 1.5 mM MPTP or MPP⁺ causes 100% and 60% ATP loss by 60 min, respectively [19], while at the same time point 90% depletion was achieved with only 0.75 mM MPDP⁺. Thus, MPDP⁺ is also the most effective in impairing the cellular energy-dependent processes. Previous studies have established that both the MAO inhibitor pargyline and the specific MAO B inhibitor deprenyl block the metabolism of MPTP to MPDP⁺ and MPP⁺ [26] and protect animals against the neurotoxicity of MPTP [27,28]. We have shown that pargyline also protects isolated hepatocytes

<table>
<thead>
<tr>
<th>Addition</th>
<th>Length of exposure (min)</th>
<th>&gt;90% dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>1.5 mM MPTP</td>
<td>95 ± 8</td>
<td>120 ± 12</td>
</tr>
<tr>
<td>1.5 mM MPDP⁺</td>
<td>68 ± 6</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>1.5 mM MPP⁺</td>
<td>190 ± 15</td>
<td>225 ± 19</td>
</tr>
</tbody>
</table>

*a* Extrapolations from time-curves based on measurements taken every 15 min

*b* Determined as the uptake of Trypan blue

*c* Data represents mean (±S D) of 4 separate incubations
Fig 2 Dose-dependent cytotoxicity (A) and depletion of ATP (B) in hepatocytes exposed to MPDP⁺. Freshly isolated rat hepatocytes were incubated as described in Methods in the absence (○) and presence of MPDP⁺ (□) at the concentrations indicated. Toxicity was measured at various time points as the uptake of Trypan blue. The data represent the mean (±S D) of 4 separate experiments.

against the toxicity of MPTP, but not that of MPP⁺ [19]. Inhibition of MAO by pargyline did not affect the extent or the rate of both hepatocyte toxicity and ATP depletion induced by MPDP⁺ (Fig. 3).

**Accumulation of MPP⁺ in hepatocytes exposed to MPTP, MPDP⁺ or MPP⁺**

Quantitative estimations of the metabolites produced after exposure of hepatocytes to MPTP showed the presence of relatively high concentrations of MPDP⁺ and comparable levels (at 30 min) of MPP⁺, PTP and MPTP-N-oxide.

Fig 3 Effect of pargyline on cytotoxicity (A) and ATP depletion (B) induced by MPDP⁺ in hepatocytes. Freshly isolated hepatocytes were either pretreated for 10 min with 10 μM pargyline (filled symbols) or not pretreated (open symbols) and then exposed to either 1.5 mM MPDP⁺ (□) or no addition (○). At the time points shown, ATP content was analyzed as described in Methods. Data represent the means (±S D) of 4 separate experiments.
Fig 4 Intracellular concentrations of MPTP, MPDP+ and MPP+ in hepatocytes incubated in the presence of 1.5 mM MPTP (A), 1.5 mM MPDP+ (B) and 1.5 mM MPP+ (C). Freshly isolated hepatocytes were incubated with the three phenylpyridine compounds and samples prepared and analyzed as described in Methods. Concentrations are expressed in relation to cell pellet protein so as to account for the diminishing proportion of intact cells remaining with time. Measurements were discontinued when cell viability dropped below 50%. Bars represent the mean concentration of MPTP (■), MPDP+ (□) and MPP+ (●) present at a given time point. Effectively, only MPP+ accumulates within the cells. The data represent means (±S.D.) of 4 separate experiments.
TABLE II
CONCENTRATIONS OF METABOLITES DETECTED IN THE MEDIUM DURING INCUBATION OF HEPATOCYTES IN THE PRESENCE OF 15 mM MPTP

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Concentration (µg/ml) at</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>MPDP⁺</td>
<td>16.5 ± 1.8</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>PTP</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>MPTP-N-oxide</td>
<td>4.6 ± 1.1</td>
</tr>
</tbody>
</table>

*Data represents mean (± SD) of 4 separate experiments

either MPDP⁺ or MPP⁺ also resulted in an increasing intracellular concentration of MPP⁺ over time (Figs. 4B and 4C). In the MPP⁺-treated cells, the levels of MPP⁺, which initially (15 min) were about 5 µg/mg, reached a concentration of about 15 µg/mg protein after 180 min. In experiments with radiolabeled MPP⁺, the radioactivity associated with the pellets could not be 'exchanged' with unlabeled MPP⁺ (data not shown). These determinations are therefore likely to reflect intracellular concentrations of this charged species rather than nonspecific binding to plasma membranes. The MPP⁺ levels in hepatocytes exposed to MPTP showed the same net increase observed in the MPP⁺ incubation mixtures although these levels were achieved after only 90 min. Incubation with MPDP⁺ led to a dramatic increase in the intracellular concentration of MPP⁺. The initial values (7.5 min) were near 10 µg/mg protein while values over 40 µg/mg protein were reached after only 60 min. Thus, the rate of MPP⁺ accumulation in the presence of MPTP or its

**Fig 5** Effect of pargyline on intracellular MPP⁺ concentration in the presence of MPTP (A), MPDP⁺ (B) and MPP⁺ (C). Freshly isolated hepatocytes were either pretreated for 10 min with 10 µM pargyline (filled symbols) or not pretreated (open symbols) and then exposed to either MPTP (●), MPDP⁺ (□) or MPP⁺ (△) all at 15 mM. At the time points shown, the intracellular concentration of MPP⁺ was determined as described in Methods. Please note the different time points used for the three different compounds. The data represent means (±SD) of 4 separate experiments.
MAO-generated metabolites correlates with their lethality to the hepatocytes. This was also true when other concentrations of the pyridine compounds were used (not shown) and when the cells were pretreated in the presence of pargyline. As shown in Fig. 5, pargyline almost completely blocked hepatocyte biotransformation of MPTP to MPP⁺, while it had no significant effect on the intracellular accumulation of MPP⁺ after exposure to either MPDP⁺ or MPP⁺.

DISCUSSION

Freshly isolated rat hepatocytes have been used in the present investigation as an in vitro model to allow a study of the relationship between MPTP metabolism and toxicity. In particular, the role played by the two MAO-generated metabolites in the events leading to cell damage has been carefully evaluated. The results of these studies clearly document that MPP⁺ is the only metabolite which increases in concentration with time in isolated hepatocytes after exposure to MPTP. The absence of significant levels of MPTP in the cells exposed to relatively high concentrations of this parent compound suggests that the lifetime of MPTP within freshly isolated rat hepatocytes is very short. The low intracellular and high extracellular levels of MPDP⁺ observed in the MPTP incubations are also noteworthy. Apparently once formed within the cell, MPDP⁺ either undergoes further oxidation to form MPP⁺, which is trapped inside the cell, or is rapidly excreted from the hepatocyte. Extracellular oxidation of MPDP⁺ is most likely responsible for the presence of MPP⁺ in the medium during cell incubations with MPTP. In a proposed model of MPTP neurotoxicity [29], MPDP⁺ has been suggested to diffuse across the membranes of glia cells, where it is produced from the metabolism of MPTP. According to the theory it would then nonenzymatically generate MPP⁺, which is taken up by terminals and cell bodies of the nigrostriatal system. Our data provide the first evidence for the feasibility of such a diffusion and transformation process with intact cells.

The toxicity of MPTP to hepatocytes is prevented by pargyline [19] suggesting that, as with the nigrostriatal toxicity [27], hepatocyte damage is mediated by a MAO-generated metabolite. This is further supported by the fact that the two products of MPTP metabolism by the microsomal mixed function oxidase system, the desmethyl and N-oxide derivatives, appear to be excreted rapidly, as they were found at barely detectable concentrations in the intact cells.

The results presented in this paper also clearly establish the hepatocytotoxic properties of MPDP⁺, the initial metabolite formed in the MAO B catalyzed oxidation of MPTP [3]. Although it is difficult to assign quantitative differences in toxicity with this type of experiment, an approximate estimation of 'toxic potencies' (Table I) reveals that MPDP⁺ is the most and MPP⁺ the least potent toxin of the group of compounds studied. The instability of MPDP⁺ has previously made it difficult to clearly assess the
events associated with its toxic properties. Since both MPTP and MPP\(^+\) are considerably less efficient in causing cell death, it seems unlikely that extracellular oxidation or disproportionation of MPDP\(^+\) is responsible for the expression of its cytotoxicity. The data does reveal, however, that MPDP\(^+\) is an excellent source of intracellular MPP\(^+\). A rough estimate of the overall extent of metabolism of MPTP vs MPDP\(^+\) indicates that MPDP\(^+\) and MPTP must have comparable access to the cells. Although MPDP\(^+\), like MPP\(^+\), is a charged species, it can also exist as the conjugate acid of the corresponding free base, i.e., as 1-methyl-4-phenyl-1,2-dihydropyridine (1,2-MPDP). The pKa of this carbon acid is likely to be lower than the corresponding N-protonated conjugate acid derived from MPTP since the nitrogen lone pair of 1,2-MPDP is sp\(^2\) hybridized whereas the lone pair of MPTP is sp\(^3\) hybridized. One pathway to account for the ready accessibility of MPDP\(^+\) to the cell, therefore, would involve passive diffusion of the lipophilic free base, i.e., the 1,2-MPDP species, into the cell. This interpretation also is consistent with the presence of MPDP\(^+\) in the media obtained from the MPTP incubation mixtures.

Similar to MPTP, the lifetime of MPDP\(^+\) in hepatocytes must be quite short since the intracellular concentrations of this dihydropyridinium species at all time points measured were very low compared to those of MPP\(^+\). MPDP\(^+\) has been reported to be a substrate for MAO B [30], although the reaction is only partially inhibited by pargyline. In the present experiments, pretreatment of the hepatocytes with pargyline had essentially no effect on the toxicity of MPDP\(^+\) or on the accumulation of MPP\(^+\). It therefore appears that MPDP\(^+\) must undergo enzymatic or biochemical oxidation reactions other than that catalyzed by MAO in liver cells.

A comparison of the intracellular concentrations of MPP\(^+\) in the various incubation mixtures shows a clear correlation with the onset of cell death. MPDP\(^+\), the most potent toxin of the three compounds studied, was also converted to MPP\(^+\) the most rapidly and extensively. In contrast, MPP\(^+\)-induced toxicity occurred only after a relatively long lag period, reflecting the slow intracellular accumulation of this charged species. Furthermore, pargyline, which prevents only MPTP-induced cytotoxicity [19], blocked hepatocyte biotransformation of MPTP to MPP\(^+\), but it had no significant effect on MPP\(^+\) accumulation after exposure to either MPDP\(^+\) or MPP\(^+\). It seems reasonable at this time to speculate that intracellular MPP\(^+\) is more closely associated with the molecular events ultimately responsible for cell death. The significance of these findings must await further investigations directed to the ultimate cause of toxicity. One factor which is likely to be involved, however, in the toxicity of MPTP in these cells is the depletion of ATP. Indeed, a generally good correlation exists between the onset of cell death, the intracellular concentration of MPP\(^+\) and the depletion of ATP. MPP\(^+\)-induced inhibition of mitochondrial respiration [10] and the energy-consuming process of mitochondrial accumulation of MPP\(^+\) [11] may both be responsible for this toxic effect. A combination of MPP\(^+\)-induced ATP consumption and decreased synthesis may therefore be the mechanism of MPTP-induced cytotoxicity.
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