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# 1-Methyl-4-phenyl-2,3-dihydropyridinium is transformed by ubiquinone to the selective nigrostriatal toxin 1-methyl-4-phenylpyridinium

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**Abstract We have studied the interaction of coenzyme Q with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its metabolites, 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) and 1-methyl-4-phenylpyridinium ($MPP^+$), the real neurotoxin to cause Parkinson's disease. Incubation of MPTP or $MPDP^+$ with rat brain synaptosomes induced complete reduction of endogenous ubiquinone-9 and ubiquinone-10 to corresponding ubiquinols. The reduction occurred in a time- and MPTP/$MPDP^+$ concentration-dependent manner. The reduction of ubiquinone induced by $MPDP^+$ went much faster than that by MPTP. MPTP did not reduce liposome-trapped ubiquinone-10, but $MPDP^+$ did. The real toxin $MPP^+$ did not reduce ubiquinone in either of the systems. The reduction by MPTP but not $MPDP^+$ was completely prevented by pargyline, a type B monoamine oxidase (MAO-B) inhibitor, in the synaptosomes. The results indicate that involvement of MAO-B is critical for the reduction of ubiquinone by MPTP but that $MPDP^+$ is a reductant of ubiquinone per se. It is suggested that ubiquinone could be an electron acceptor from $MPDP^+$ and promote the conversion from $MPDP^+$ to $MPP^+$ in vivo, thus accelerating the neurotoxicity of MPTP.**



*Key words:* 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 1-Methyl-4-phenyl-2,3-dihydropyridinium; Coenzyme Q; Ubiquinone; Monoamine oxidase; Rat brain synaptosome; Parkinson's disease

## 1. Introduction

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces Parkinsonism in humans [1] and experimental animals [2–5]. The mechanism of neurotoxicity of MPTP has been investigated extensively in the 1980s. Studies have elucidated that MPTP is metabolized by type B monoamine oxidase (EC 1.4.3.4, MAO-B) to the initial two-electron oxidation product, 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$), which undergoes further oxidation to the ultimate four-electron oxidation product, 1-methyl-4-phenylpyridinium ($MPP^+$) [6], which is sequestered in dopaminergic neurons by the high-affinity dopamine uptake pump [7]. The toxicity of $MPP^+$ apparently occurs by selective inhibition of NADH-ubiquinone oxidoreductase (complex I) of the mitochondrial respiratory chain, resulting in ATP depletion and finally cell death [8,9]. An alternative mechanism of MPTP toxicity may involve the generation of toxic active oxygen species [10,11]. However, it is still unclear how $MPDP^+$ is transformed to $MPP^+$ in vivo.

Ubiquinone, which is also known as coenzyme Q, is a lipid-soluble compound composed of a redox active quinoid moiety and a hydrophobic chain. Coenzyme Q, the electron acceptor for complexes I and II, acts as an electron and proton carrier in mitochondrial and bacterial electron transport linked to ATP synthesis. In addition, ubiquinol, the reduced form of ubiquinone, functions as a radical-scavenging antioxidant preventing the chain initiation and/or propagation of lipid peroxidation in biological membranes and in low-density lipoprotein. Its high degree of hydrophobicity and its widespread occurrence in biological membranes suggest an important role of ubiquinol in cellular defense against oxidative damage [12]. So, coenzyme Q plays an important role in the two areas implicated in the neurotoxicity of MPTP, mitochondrial dysfunction and oxidative damage. Despite the extensive studies on MPTP metabolism and neurotoxicity and the essential role of coenzyme Q in the electron transport chain, the effect of coenzyme Q on MPTP metabolism has not been investigated. We now show for the first time, to our knowledge, that MPTP through its metabolite by MAO-B, $MPDP^+$, reduces ubiquinone to its reduced form. The results indicate that coenzyme Q may play an important role in transformation of $MPDP^+$ to $MPP^+$ in vivo.

## 2. Materials and methods

### 2.1. Materials

MPTP hydrochloride, pargyline (*N*-benzyl-*N*-methyl propargylamine), and Chelex 100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). $MPDP^+$ perchlorate and $MPP^+$ iodide were purchased from Research Biochemicals International (Natick, MA, USA). Phosphatidylcholine (14:0) and sodium borohydride were from Wako Pure Chemical Co. (Osaka, Japan). Ubiquinone-9 and ubiquinone-10 were kindly supplied by Eisai Co. (Tokyo, Japan). Ubiquinol-9 and ubiquinol-10 were obtained by reduction of corresponding quinones with sodium borohydride. All solvents used for high performance liquid chromatography (HPLC) analysis were from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals used were of analytical grade.

### 2.2. Experimental animals and isolation of synaptosomes

Male Wistar rats weighing 180–200 g were purchased from Clea Co. (Tokyo, Japan) and acclimatized for 1 week before use. Synaptosomes were prepared from the whole brain of rats by the discontinuous density gradient centrifugation method of Dodd et al. [13].

### 2.3. Incubation of synaptosomes with MPTP, $MPDP^+$ or $MPP^+$

Synaptosomes (1 mg protein/ml) were incubated with MPTP ($MPDP^+$ or $MPP^+$) at various concentrations under air at 37°C in 10 mM phosphate-buffered saline (PBS, pH 7.4). PBS was pre-treated

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*Abbreviations:* EC, electrochemical; HPLC, high performance liquid chromatography; MAO, monoamine oxidase; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium; $MPP^+$, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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with Chelex 100 to remove traces of metal ions. The suspension of synaptosomes was pre-equilibrated for 5 min at 37°C before the addition of other substances. In the experiment with the MAO-B inhibitor, pargyline was added to the suspensions at the same time as MPTP.

*2.4. Incubation of liposome-trapped coenzyme Q with MPTP, $MPDP^+$ or $MPP^+$*

The liposome was prepared as follows. Phosphatidylcholine and ubiquinone-10 were dissolved in chloroform and the solution was placed in a flask. The solvent was removed to obtain a thin film. An appropriate amount of PBS was added and the film was slowly peeled off by shaking to obtain a white, milky liposome solution. MPTP, $MPP^+$ or $MPDP^+$ was added to a portion of the liposome solution in a reaction vessel, mixed and incubated under air at 37°C. Aliquots were taken out at indicated times and injected directly on an HPLC system (see Section 2.5) to measure the content of ubiquinone and ubiquinol.

*2.5. Extraction and measurement of ubiquinone and ubiquinol*

Synaptosomes (0.4 mg protein) were separated from the reaction medium, resuspended in 0.2 ml water, and frozen and thawed twice using liquid nitrogen. Lipophilic substances including coenzyme Q were extracted by chloroform/methanol (2:1, v/v). The lower phase was separated out and evaporated under a stream of nitrogen. The residue was redissolved in methanol for HPLC analysis. The HPLC system was the same as used previously [14].

*2.6. Measurement of protein content*

Protein content was determined by the BCA protein assay kit according to the maker's instruction (Pierce, Rockford, IL, USA).

## 3. Results and discussion

*3.1. HPLC separation of coenzyme Q*

As shown in Fig. 1, synaptosomal ubiquinones and ubiquinols were separated and quantitated by HPLC with UV and electrochemical (EC) detectors. Two kinds of coenzyme Q, ubiquinone-9 and ubiquinone-10, exist in rat brain synaptosomes. The content of ubiquinone-9 was twice that of ubiquinone-10. In control synaptosomes, the level of ubiquinols was very low. After incubation of synaptosomes with MPTP, the peaks of ubiquinones disappeared and the peaks of ubiquinols increased significantly.

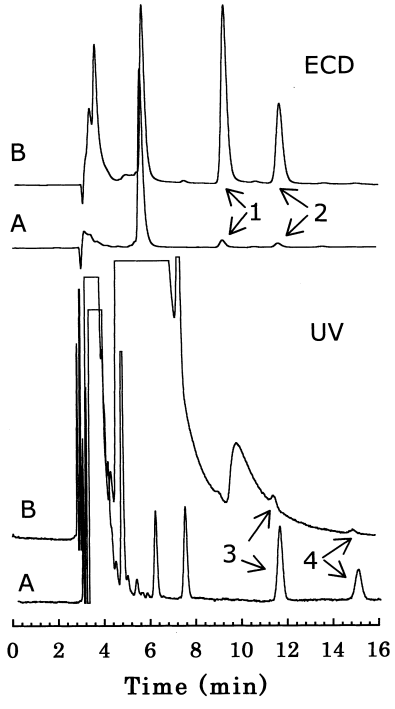



Fig. 1. HPLC analysis of ubiquinones and ubiquinols in extracts from rat brain synaptosomes with UV and EC detectors. A: Control synaptosomes. B: Synaptosomes incubated with MPTP (1 mM) for 30 min at 37°C. Peaks 1, 2, 3, and 4 stand for ubiquinol-9, ubiquinol-10, ubiquinone-9, and ubiquinone-10, respectively.

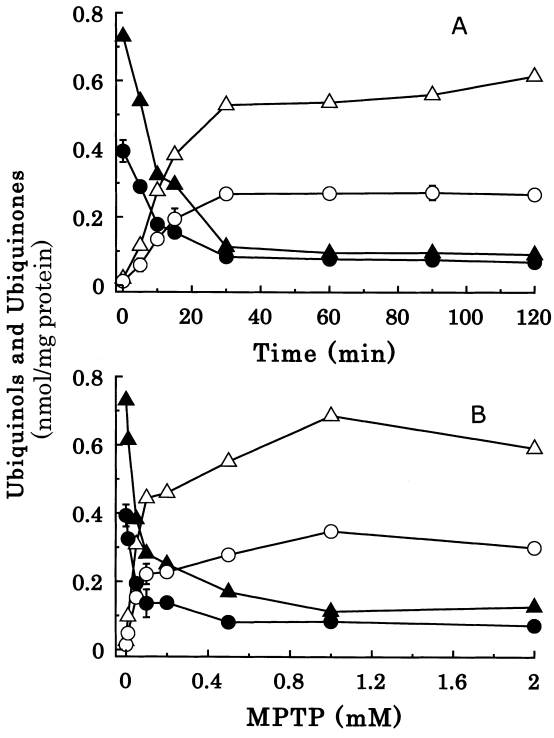



Fig. 2. Reduction of endogenous ubiquinones to their reduced forms by MPTP in rat brain synaptosomes. A: Time-dependent study. Rat brain synaptosomes (1 mg protein/ml) were incubated in PBS (10 mM, pH 7.4) in the presence of 0.5 mM MPTP at 37°C under air for 120 min. Aliquots were removed at various time intervals and levels of ubiquinols and ubiquinones were measured by HPLC. B: Concentration-dependent study. Rat brain synaptosomes (1 mg protein/ml) were incubated with MPTP at various concentrations in PBS (10 mM, pH 7.4) at 37°C under air for 30 min. ▲, ubiquinone-9; △, ubiquinol-9; ●, ubiquinone-10; ○, ubiquinol-10. Data are means ± S.D. of three independent determinations.

*3.2. Interaction of MPTP with coenzyme Q*

The reduction of ubiquinone to ubiquinol by MPTP in the synaptosomes occurred time-dependently (Fig. 2A). The rate of reducing endogenous ubiquinone-9 (0.018 nmol/mg protein/min) was twice that of ubiquinone-10 (0.009 nmol/mg protein/min). Almost all the endogenous ubiquinone-9 and ubiquinone-10 was depleted in 30 min, as observed by the concomitant production of ubiquinol-9 and ubiquinol-10, respectively. The levels of ubiquinols were very stable afterwards during the 2-h incubation. Fig. 2B shows that the reduction of synaptosomal ubiquinones proceeded MPTP concentration-dependently. There were two phases of MPTP concentration-dependent reduction of ubiquinones, and at the concentration of 1 mM, the reduction reached a plateau. When exogenous ubiquinone-10 (20 times higher than the level of endogenous ubiquinone-10) was added to synaptosomes, it could also be reduced quantitatively (data not shown).

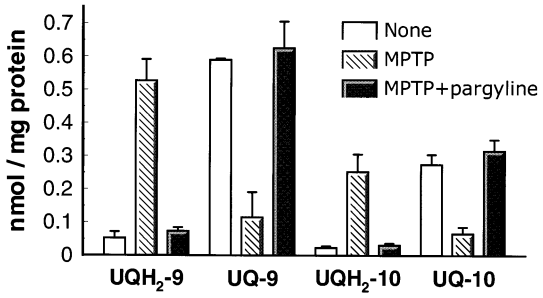



Fig. 3. Effect of pargyline on the reduction of ubiquinones by MPTP in rat brain synaptosomes. Rat brain synaptosomes (1 mg protein/ml) were incubated with MPTP (1 mM) in the presence or absence of pargyline (1 μM) in PBS (10 mM, pH 7.4) at 37°C under air for 3 h. Aliquots were taken and levels of ubiquinols and ubiquinones were measured by HPLC. UQ-9, ubiquinone-9; $UQH_2$-9, ubiquinol-9; UQ-10, ubiquinone-10; $UQH_2$-10, ubiquinol-10. Data are means ± S.D. of three independent determinations.

To determine whether MPTP reduces ubiquinone by itself or through its metabolites, the following experiments were carried out. First, the effect of pargyline, a MAO-B inhibitor, on the reduction of synaptosomal ubiquinone by MPTP was studied. MAO-B has a crucial role in MPTP-induced degeneration of the nigrostriatal dopaminergic neuronal pathway. Chiba et al. discovered that rodent brain preparations could metabolize MPTP to $MPP^+$ in vitro [6] and that potent inhibitors of MAO-B could inhibit the $MPP^+$ formation from MPTP, suggesting MAO-B is responsible for the oxidative metabolism of MPTP. In vivo studies showed that inhibitors of MAO-B, but not MAO-A, could protect monkeys and mice from the neurotoxicity of MPTP, indicating that its in situ oxidation by MAO-B accounts for the cytotoxicity in the brain [15,16]. As shown in Fig. 3, after 3 h of treatment with MPTP, synaptosomal ubiquinone-9 and ubiquinone-10 exist in their reduced forms. Strikingly, no reduction of ubiquinones was observed in synaptosomes treated with pargyline and MPTP together. In agreement with the cytotoxicity study, MAO-B plays a crucial role in the reduction. Second, MPTP was incubated with liposome-trapped ubiquinone-10. It can be seen from Fig. 4 that MPTP did not react or reduce liposome-trapped ubiquinone-10. In addition, it was confirmed that $MPP^+$, the real neurotoxin and the final metabolite of MPTP, did not possess the property to reduce ubiquinone in either rat brain synaptosomes or liposomes (data not shown).

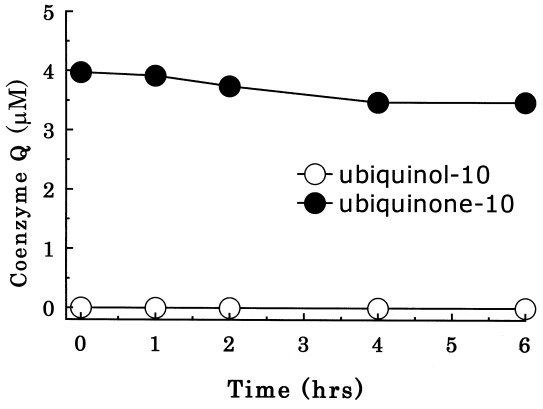



Fig. 4. Effect of MPTP on liposome-trapped ubiquinone. Liposomes of 14:0 phosphatidylcholine (2.83 mM) containing ubiquinone-10 (4.0 μM) were incubated with MPTP (2 mM) in PBS (10 mM, pH 7.4) at 37°C under air. Aliquots were removed at various time intervals and levels of ubiquinone-10 (●) and ubiquinol-10 (○) were measured by HPLC.

### 3.3. Interaction of $MPDP^+$ with coenzyme Q

We further investigated the interaction between coenzyme Q and $MPDP^+$. In rat brain synaptosomes, $MPDP^+$ reduced ubiquinone-9 and ubiquinone-10 to their reduced forms time-dependently and the reduction was much faster than that by MPTP (Fig. 5A). $MPDP^+$ led to complete reduction of ubiquinones to their reduced forms in 5 min. The reduction also occurred $MPDP^+$ concentration-dependently (Fig. 5B). $MPDP^+$ at concentrations greater than 0.05 mM reduced all the synaptosomal ubiquinone. Not only did $MPDP^+$ reduce synaptosomal ubiquinone, but it also reduced liposome-trapped ubiquinone (Fig. 6), which indicates that $MPDP^+$ directly reduced ubiquinone. The reduction of liposomal ubiquinone was $MPDP^+$ concentration- and time-dependent. These results indicate that MPTP reduces synaptosomal ubiquinone through its metabolism by MAO-B to $MPDP^+$. In addition, MPTP and $MPDP^+$ also reduced ubiquinones in rat liver mitochondria (unpublished data) in a similar way as in the synaptosomes.

$MPDP^+$ is chemically reactive and has a tendency to undergo disproportionation at high concentrations and at alkaline

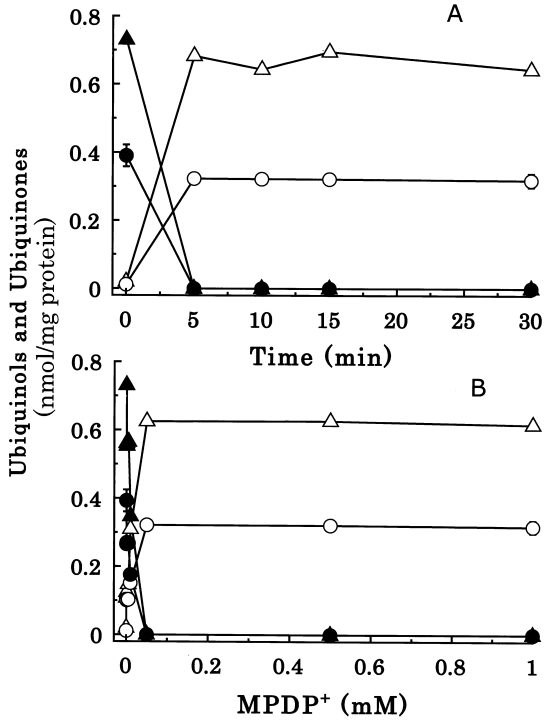



Fig. 5. Interaction of synaptosomal ubiquinones with $MPDP^+$. A: Time-dependent study. Rat brain synaptosomes (1 mg protein/ml) were incubated in PBS (10 mM, pH 7.4) in the presence of 0.1 mM $MPDP^+$ at 37°C under air. Aliquots were taken at various time intervals and levels of ubiquinols and ubiquinones were measured by HPLC. B: Concentration-dependent study. Rat brain synaptosomes (1 mg protein/ml) were incubated with $MPDP^+$ at various concentrations in PBS (10 mM, pH 7.4) at 37°C under air for 5 min. ▲, ubiquinone-9; △, ubiquinol-9; ●, ubiquinone-10; ○, ubiquinol-10. Data are means ± S.D. of three independent determinations.

pH values. But this does not appear to be a physiologically significant source of $MPP^+$, because at neutral pH the disproportionation hardly proceeds. In fact, at the early stage of the study on the neurotoxicity of MPTP it was found that the decrease of $MPDP^+$ was accelerated in the presence of rat brain mitochondria [17]. Because both heat-denatured mitochondria and addition of the MAO-B inhibitor pargyline did not show any effect on the observed rate of decomposition of $MPDP^+$ to $MPP^+$, it was concluded that the conversion was not enzyme-mediated [17,18], and the reason why brain mitochondrial preparations enhanced decomposition of $MPDP^+$ is still unknown, although it was later argued that the decomposition of $MPDP^+$ to $MPP^+$ may be catalyzed by metal chelates [19] and neuromelanin [20]. Our observation should shed new light on the metabolism of MPTP in vivo. The results enable us to propose the metabolism of MPTP as shown in Scheme 1. MPTP is oxidized by MAO-B to $MPDP^+$, which can transfer its two electrons to the oxidized form of coenzyme Q present ubiquitously in biological membranes, and thus enhance the production of $MPP^+$. Coenzyme Q could be a critical factor in the mechanism of toxicity of MPTP in this respect. This may raise the question whether it is proper or not to use coenzyme Q to ameliorate the toxicity of MPTP because recently coenzyme Q-10 has been proposed to improve a MPTP-induced Parkinsonism model [21,22].

It has been suggested that a free radical-mediated mechanism could be involved in MPTP toxicity (see reviews [10,11]). MPTP produces hydrogen peroxide by the action of MAO-B. MPTP, $MPDP^+$ and $MPP^+$ themselves catalyze superoxide radical production under certain conditions. However, it has been reported that both MPTP and $MPDP^+$ inhibited lipid peroxidation in isolated hepatocytes [23], liver homogenates [24] and brain homogenates [24]. The reason has been mysterious. Our finding that MPTP and $MPDP^+$ are capable of reducing ubiquinone to ubiquinol can fully explain the previous observation. It is obvious that the interaction between $MPDP^+$ and ubiquinone will significantly increase the total antioxidant potency because ubiquinol serves as a potent antioxidant in biological membranes. We also observed that in the presence of MPTP or $MPDP^+$, but not $MPP^+$, the depletion of α-tocopherol and production of phospholipid hydroperoxides in synaptosomes were completely inhibited during oxidation induced by peroxyl radical and the effect is ascribed to the antioxidant activity of ubiquinol constantly reduced from ubiquinone by MPTP and $MPDP^+$ (Shi and Niki, unpublished data).

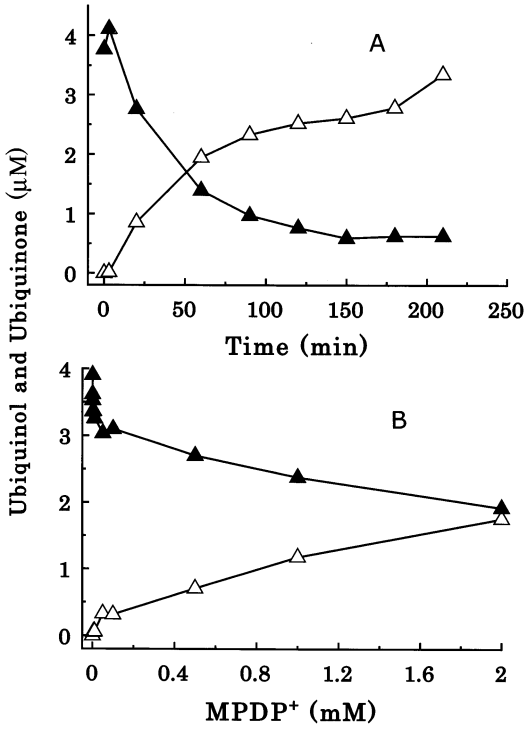
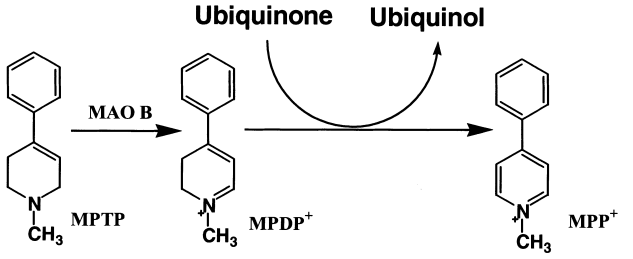



Fig. 6. Interaction of liposome-trapped ubiquinone-10 with $MPDP^+$. A: Time-dependent study. Liposomes of 14:0 phosphatidylcholine (2.83 mM) containing ubiquinone-10 (4.0 μM) were incubated with $MPDP^+$ (2 mM) in PBS (10 mM, pH 7.4) at 37°C under air. Aliquots were taken at various time intervals and levels of ubiquinol and ubiquinone were measured by HPLC. B: Concentration-dependent study. Liposomes containing ubiquinone-10 were incubated with $MPDP^+$ in PBS (10 mM, pH 7.4) at 37°C under air for 1 h. ▲, ubiquinone-10; △, ubiquinol-10. The result is a representative of duplicate determinations.




Scheme 1. The proposed metabolism of MPTP in vivo.

Attempts to prevent the toxic consequences of MPTP exposure in vivo via the use of antioxidants have been carried out. Substances with antioxidant activity such as cytosine [25], *Ginkgo biloba* [26] and melatonin [27,28] have been used to prevent neurotoxicity of MPTP in animal models. In this respect, it seems the improved antioxidant capacity by the interaction of $MPDP^+$ with coenzyme Q would decrease the toxicity of MPTP. This leads coenzyme Q to play a contradictory role in the toxicity of MPTP. Coenzyme Q accelerates the toxicity of MPTP by enhancing the transformation of $MPDP^+$ to $MPP^+$, while it may ameliorate the toxicity of MPTP by acting as an efficient antioxidant.

In conclusion, this is the first observation that ubiquinone can be reduced to its reduced form by MPTP and its metabolite $MPDP^+$. This provides a new route of the metabolism of MPTP in vivo. MPTP is metabolized to $MPDP^+$ by MAO-B and $MPDP^+$ is transformed to $MPP^+$ by reducing ubiquinone. In this respect, coenzyme Q may play a critical role in the toxicity of MPTP.

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