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A REDOX REACTION BETWEEN MPP^+ AND $MPDP^+$ TO PRODUCE SUPEROXIDE RADICALS DOES NOT IMPAIR MITOCHONDRIAL FUNCTION

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Abstract—Rat brain mitochondria were incubated with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its two metabolites (1-methyl-4-phenyl-2,3-dihydropyridium ($MPDP^+$) and 1-methyl-4-phenylpyridinium (MPP^+), and O_2 uptake was assessed. MPP^+ (500 and 1000 μM) inhibited state 3 and state 4 respiration with a reduction in the respiratory control ratio (RCR). In the presence of MPTP or $MPDP^+$ (100–1000 μM) no inhibition of mitochondrial function occurred. Incubation with MPP^+ (100–1000 μM) in combination with equimolar concentrations of $MPDP^+$ or MPTP (100–1000 μM) did not increase the inhibition of mitochondrial function produced by MPP^+ alone. Inhibition of mitochondrial function produced by MPP^+ (500 μM) was not reduced by incorporation of superoxide dismutase (SOD) (50–1000 units/mL). However, the RCR in the presence of 500 μM MPP^+ and 1000 units/mL SOD was not different from control values. SOD did not prevent the inhibition of state 3 and state 4 respiration produced by the combination of MPP^+ and $MPDP^+$. The results suggest that a redox reaction between MPP^+ and $MPDP^+$ to generate superoxide radicals does not contribute to the impairment of mitochondrial function produced by MPTP administration.

Administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to man and other primate species results in selective toxicity to the dopamine containing cells in the zona compacta of the substantia nigra and produces parkinsonian motor deficits [1–4]. Toxicity appears to be due to the formation of the pyridinium species, 1-methyl-4-phenylpyridinium (MPP^+). Thus, MPP^+ but not MPTP is highly toxic to nigral cells in culture [5], and to nigral dopamine cells on direct infusion into brain [6]. The formation of MPP^+ occurs as a result of the metabolism of MPTP by monoamine oxidase B (MAO B). Subsequently, MPP^+ is transported by the dopamine uptake process to accumulate within dopaminergic neurones. The toxicity of MPP^+ apparently occurs as a result of the impairment of mitochondrial function. *In vitro*, MPP^+ accumulates within mitochondria by an active transport process occurring across the mitochondrial membrane and results in oxidative stress due to inhibition of complex I of the respiratory chain [7, 8]. The same mechanism may occur *in vivo* since administration of MPTP to mice inhibits complex I activity and state 3 and state 4 respiration in mitochondrial preparations [9]. However, the concentrations of MPP^+ required *in vitro* to inhibit mitochondrial function are high and there are doubts whether comparable levels can be achieved *in vivo* [10].

An alternative mechanism of MPTP toxicity may involve the generation of toxic oxygen species. Initially this would appear unlikely since both MPTP and MPP^+ have electrochemical potentials in excess of -1.0 V, which makes the possibility of bioreduction remote [11, 12]. However, MPP^+ may not be the only species contributing to MPTP toxicity. Indeed, the initial step in the metabolism of MPTP by MAO B is the formation of 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) which then disproportionates to produce MPP^+ and MPTP. Recently, Rossetti *et al.* [13] suggested that a redox reaction occurred between the MPTP metabolites MPP^+ and $MPDP^+$, to generate superoxide radicals. Using mouse brain mitochondrial preparations, the inclusion of MPTP caused an electron spin resonance signal (ESR), indicative of radical formation. The signal was reduced by the selective MAO B inhibitor deprenyl, but not by the MAO A inhibitor clorgyline. The ESR signal was suppressed by the inclusion of SOD, suggesting the formation of superoxide radicals. In aqueous solution neither MPP^+ nor $MPDP^+$ alone generated an ESR signal. However, the combination of $MPDP^+$ and MPP^+ produced an ESR signal which increased in intensity with time.

It is not known whether the generation of superoxide radicals contributes to the toxicity of MPTP (or MPP^+) to the mitochondrial respiratory chain, or whether it represents a potential alternative mechanism of toxicity. In the present study we have investigated the effects of MPTP, $MPDP^+$ and MPP^+ alone and in combination on mitochondrial respiratory function, and the ability of superoxide dismutase to inhibit the effects of MPP^+ alone and in combination with $MPDP^+$ and MPTP.

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MATERIALS AND METHODS

Preparation of mitochondria. Mitochondria were isolated from the brains of male Wistar rats (200–400 g; Bantin and Kingman, Hull, U.K.). The method used was a modification of that of Lai and Clark [14]. Rats were killed by cervical dislocation and decapitation and the brains were removed rapidly. The whole brain, minus the cerebellum, was chopped up using fine stainless steel scissors into 1–2 mm sized cubes in 10 volumes of ice-cold (4°) 0.32 M sucrose containing 10 mM Tris–HCl and 1 mM EDTA (pH 7.4) (isolation medium). The tissue was drained, placed in 30 mL of fresh isolation medium and carefully homogenized using a hand-held 40 mL glass/glass Dounce tissue homogenizer. The homogenate was spun at 3500 *g* for 5 min at 4° using a Sorvall RC3B centrifuge. The resulting supernatant was then spun at 10,000 *g* for 10 min at 4° in a Sorvall RC5B centrifuge. The supernatant was discarded, the pellet resuspended in 5.0 mL of cold isolation medium and gently homogenized in a 10 mL PTFE/glass hand-held homogenizer. To this homogenate was added 25 mL of ice-cold (4°) 12% Ficoll (w/v) in Ficoll medium {225 mm mannitol, 75 mM sucrose, 1 mM EDTA and 5 mM MOPS (3-[*N*-morpholino]propanesulphonic acid); pH 7.4} with gentle mixing. The preparation was divided into two 38.5 mL thick-walled polycarbonate centrifuge tubes. To each was added 15 mL of 7.5% (w/v) Ficoll in Ficoll medium layered on to the top of the original suspension using a pasteur pipette to form a discontinuous Ficoll gradient. The samples were then spun at 100,000 *g* for 30 min at 4° in a Kontron T1055 ultracentrifuge.

The resulting mitochondrial pellets were resuspended in 2.5 mL of isolation medium and gently homogenized in a 10 mL glass/PTFE homogenizer. The homogenate was spun at 10,000 *g* for 10 min at 4° using the Sorvall RC5B centrifuge, and the resulting mitochondrial pellet was gently dispersed in 5.0 mL of cold isolation medium containing 0.5 mg/mL BSA and then finally resuspended using a glass/PTFE homogenizer. The preparations were maintained on ice at approximately 4° until required. The protein content of the preparations was measured by the technique of Lowry *et al.* [15].

Measurement of mitochondrial respiration. All measurements of O_2 uptake were made using a Clarke-type oxygen electrode (Yellow Spring Instruments, OH, U.S.A.), adapted with 1.0 mL incubation chambers, contained within a water-jacketed circulatory system linked to a water cooler and flow heater (Grant Instruments, Cambridge, U.K.). All measurements were carried out at 24° with constant stirring. The system was calibrated using 10 μL of catalase (230 units/mL; Sigma Chemical Co., Poole, U.K.) and 5 μL of H_2O_2 (1 in 250 dilution of 30% solution) in 1.0 mL of nitrogen saturated respiration medium (25 mM sucrose, 75 mM mannitol, 95 mM KCl, 0.05 mM EDTA, 5 mM K_2HPO_4 and 20 mM Tris–HCl at pH 7.4). Measurements of O_2 uptake were made using 100 μL of a mitochondrial suspension, 10 μL of a 250 mM solution of pyruvate and 10 μL of a 250 mM solution of malate as respiratory substrates with air saturated respiration medium, to give a final volume of 1.0 mL. The resting O_2 uptake (state 4) was then measured. Following the introduction of 10 μL of a 25 mM solution of ADP (Sigma) using a Hamilton Microliter syringe, state 3 O_2 uptake was measured. After a few minutes, O_2 uptake returned to state 4 respiration and the addition of ADP was repeated. Total incubation time in each case was 20 min. Respiratory control ratios (RCR) were calculated from the ratio of state 3 to state 4 O_2 uptake.

Basal state 3 O_2 uptake values ranged from 13.19 to 36.80 nmol O_2/mg protein/min (N = 40). Because of the variation in basal data over the course of the experiments, the data are expressed as a percentage of the appropriate mean basal control values. Measurement of state 3 and state 4 O_2 uptake gave a mean RCR of 3.3 ± 0.1 (N = 40).

Incubation of mitochondrial preparation with MPTP and its metabolites. A solution (100 mM) of MPTP hydrochloride (kindly provided by Schering, Berlin, F.R.G.), $MPDP^+$ perchlorate and MPP^+ hydrochloride (Semat, St Albans, U.K.) was prepared in respiration medium. Aliquots (1–10 μL) of this solution gave final concentrations of 100–1000 μM. MPTP was dissolved initially in 1.0 mL of absolute ethanol before dilution with respiration medium. In the incubations, the volume of respiration medium used was adjusted appropriately so the final volume remained 1.0 mL.

Initially, MPTP, $MPDP^+$ and MPP^+ were incubated individually at concentrations of 100–1000 μM to determine the effects upon mitochondrial state 3 and state 4 respiration. Subsequently, MPP^+ was incubated with equimolar concentrations of $MPDP^+$ or MPTP, with mitochondrial preparations. The compounds were added separately from the stock solutions at the start of incubation in equimolar concentrations of 100, 500 or 1000 μM. The effects of these combinations on mitochondrial state 3 and state 4 respiration was measured.

In some experiments, the effect of $MPDP^+$ (100–1000 μM) alone or in the presence of MPP^+ (100 μM) on mitochondrial function was examined in a photographic dark-room. Preparation of solutions and all manipulations were carried out with minimum exposure to light.

Spectrophotometric analysis of mitochondrial preparations containing MPTP, $MPDP^+$ and MPP^+. Aqueous solutions of MPTP, $MPDP^+$ or MPP^+ (100 μM) were scanned between 190 and 500 nm in quartz cells using a Shimadzu UV2101 dual beam spectrophotometer. Characteristic absorbance peaks were observed as follows: MPTP 242 nm; MPP^+ 292 nm; $MPDP^+$ 292 and 345 nm.

In subsequent experiments, 1000 μM MPTP, $MPDP^+$ or MPP^+ were incubated for 20 min with mitochondrial suspensions containing 1 mg mitochondrial protein, 2.5 mM malate, 2.5 mM pyruvate and respiration medium to a final volume of 1.0 mL at room temperature with constant shaking. To each incubation was then added 100 μL of a 1% (v/v) solution of Triton X-100 to solubilize the suspension and clear the solution. A 100 μL aliquot was then removed, diluted with 900 μL distilled water and immediately scanned between

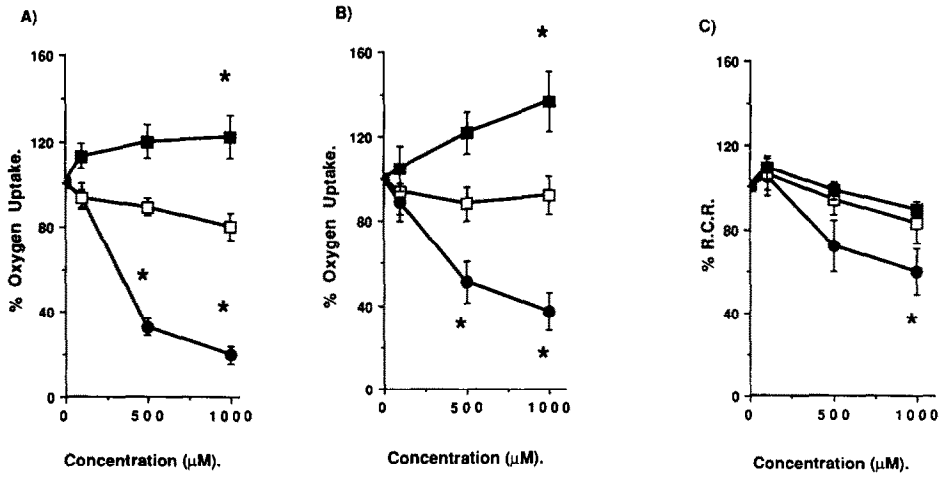

Fig. 1. The effect of MPTP, MPDP+ and MPP+ (100–1000 μM) on (A) state 3, (B) state 4 respiration and (C) respiratory control ratio (RCR) of rat mitochondrial preparation. Data are presented as the mean ± SEM for 3 individual experiments. * $P < 0.05$ compared to basal levels; Student's t-test. MPP+ (●); MPDP+ (□); MPTP (■).

190 and 500 nm against an appropriate reference sample. Scans were analysed using a "peak pick" programme for comparison of absorbance maxima.

Incubation of mitochondrial preparations with SOD. Solutions of bovine erythrocyte SOD (Sigma) were prepared at a concentration of 5000 units/mL in respiration medium and kept frozen until required. Aliquots of the stock solution (10–200 μL) were used to produce final concentrations of SOD of 50–1000 units/mL in the incubation chamber. The amount of respiration medium was adjusted appropriately to maintain the final volume at 1.0 mL.

The effect of SOD (50–1000 units/mL) upon the mitochondrial state 3 and state 4 respiration was assessed, and the experiment repeated in the presence of 500 μM MPP+. SOD (50 and 100 units/mL) was incubated also with mitochondria following the addition of MPTP, MPDP+ or MPP+ (100, 500 and 1000 μM) alone or following the addition of combinations of MPP+ with MPDP+ or MPP+ with MPTP (100, 500 and 1000 μM). The levels of state 3 and state 4 respiration were measured.

RESULTS

Effect of MPTP, MPDP+ or MPP+ on mitochondrial function

The incorporation of MPTP (100–1000 μM) into the mitochondrial preparations did not inhibit state 3 or state 4 respiration (Fig. 1). At the highest concentration employed (1000 μM) there was an increase in both state 3 and 4 respiration. The RCR was unaffected by the inclusion of MPTP. Inclusion of MPDP+ (100–1000 μM) did not alter state 3 or state 4 respiration or alter the RCR for the mitochondrial preparations (Fig. 1). When the incubation of MPDP+ was repeated in the absence of light, no inhibition of state 3 respiration was observed at 100 or 500 μM. At 1000 μM there was a small reduction of state 3 respiration (74% of basal oxygen uptake; $P < 0.05$). Inclusion of MPDP+ (100–1000 μM) in the dark did not inhibit state 4 respiration and there was no consistent reduction of RCR (data not shown).

In contrast, the incorporation of MPP+ (100–1000 μM) caused a concentration-related inhibition of state 3 and state 4 respiration (Fig. 1). There was a corresponding decrease in RCR which was significant at the highest concentration of 1000 μM.

Effect of combinations of MPP+ and MPDP+ or MPTP

Incorporation of MPP+ (500–1000 μM) caused an inhibition of state 3 and state 4 respiration (Table 1). In combination with MPTP (100–1000 μM), no further increase in the inhibition of mitochondrial respiration was observed (Table 1). Similarly MPP+ (500 μM) combined with MPDP+ (100–1000 μM) did not produce an effect greater than that produced by MPP+ alone. Also, in the light or dark the addition of MPDP+ (100–1000 μM) to MPP+ (100 μM) produced no greater inhibition of state 3 or 4 respiration or RCR than produced by MPP+ (100 μM) alone (data not shown).

Spectrophotometric analysis of mitochondrial preparations containing MPTP, MPDP+ and MPP+

After incubation of MPTP (1000 μM) with mitochondrial preparations the absorbance at 242 nm showed that only 80% of the original material remained. A small absorbance maximum was observed at 345 nm, equivalent to 13 μM MPDP+, but no peak was observed at 292 nm characteristic of MPP+ (Fig. 2). After incubation of 1000 μM MPDP+ with mitochondrial preparations approximately 95% of the initial materials remained. These incubations showed an absorbance at 292 nm but this was no greater than that occurring prior to incubation. Following incubation of 1000 μM MPP+ with mitochondrial preparations 79% remained as

Table 1. Effect of MPP^+ alone and with equimolar $MPDP^+$ or MPTP on mitochondrial state 3 and state 4 oxygen uptake and respiratory control ratio (RCR)

Concentration (μM)	MPP^+ alone			MPP^+ with $MPDP^+$			MPP^+ with MPTP		
	State 3	State 4	RCR	State 3	State 4	RCR	State 3	State 4	RCR
0				23.90 ± 1.10†	7.38 ± 0.37†	3.33 ± 0.10†			
100	16.59 ± 1.09	5.86 ± 0.61	2.98 ± 0.26	15.47 ± 1.77	5.44 ± 0.98	3.27 ± 0.41	18.41 ± 1.51	7.04 ± 0.85	2.67 ± 0.12
500	5.87 ± 0.63*	3.34 ± 0.63*	2.03 ± 0.34*	7.38 ± 0.98*	2.72 ± 0.50*	2.91 ± 0.24	11.81 ± 0.79*	4.59 ± 0.53*	2.65 ± 0.20
1000	3.52 ± 0.62*	2.40 ± 0.62*	1.71 ± 0.32*	2.67 ± 0.37*	1.49 ± 0.29*	2.60 ± 0.80	7.52 ± 0.64*	4.02 ± 0.38*	1.89 ± 0.05*

RCR values were calculated as the ratio of state 3 over state 4 respiration. Units of O_2 uptake are nmol O_2/mg protein/min. Data are expressed as mean ± SEM for 3 individual experiments.

* $P < 0.05$ compared to basal values; Student's t-test.

† Combined group mean values for controls run throughout the course of the experiments (N = 40).

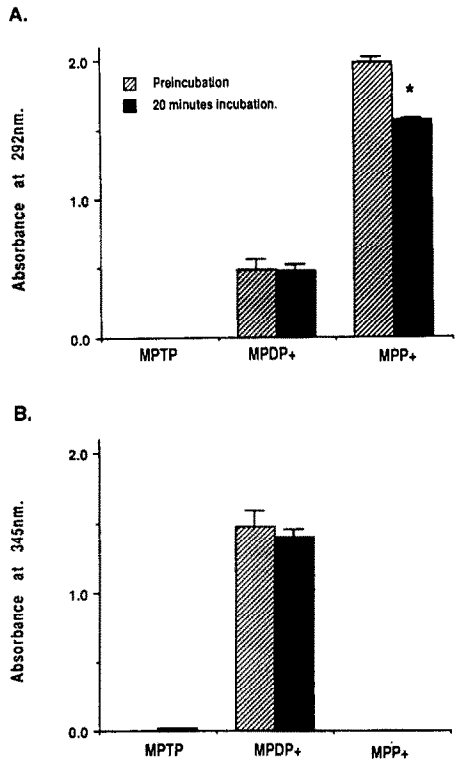

Fig. 2. Spectrophotometric analysis of solutions containing 1000 μM MPTP, $MPDP^+$ or MPP^+ before and after 20 min incubation in mitochondrial preparations showing absorbances at (A) 292 nm and (B) 345 nm. Results expressed as mean ± SEM for 3 individual experiments. Absorbance at 242 nm was obscured by quenching caused by components of the mitochondrial incubation and so could not be assessed accurately. * $P < 0.05$ compared to absorbance values before incubation; Student's t-test.

judged by absorbance at 292 nm. No peak was observed at 345 nm.

Effect of superoxide dismutase on mitochondrial respiration

The incorporation of SOD (50–1000 units/mL) had no effect on state 3 or state 4 respiration or on the RCR of the mitochondrial preparations.

Incorporation of MPP^+ (500 μM) into mitochondrial preparations inhibited state 3 and state 4 respiration and depressed the RCR (Fig. 3).. Inclusion of SOD (50–100 units/mL) caused a partial reversal of the inhibition of state 3 respiration caused by MPP^+. However, the inhibition of state 4 respiration was unaffected by the inclusion of SOD. At 50 and 100 units/mL, SOD had no effect on the depression of RCR caused by MPP^+. However, at higher concentrations (250–1000 units/mL) SOD reversed the effect of MPP^+ on RCR, which was not different from that observed in control preparations.

The addition of SOD (50 or 100 units/mL) raised state 3 and state 4 respiration in the presence of 100 μM MPP^+ (Fig. 4). However, the RCR value was still depressed. At higher MPP^+ concentrations

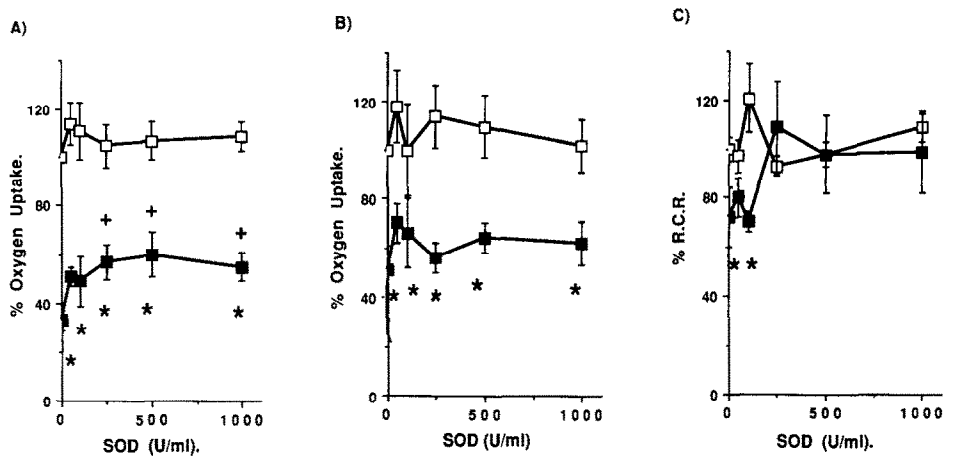

Fig. 3. The effect of SOD (50–1000 units/mL) on MPP+ induced inhibition of (A) mitochondrial state 3, (B) state 4 respiration and (C) respiratory control ratio (RCR). Data are presented as the mean ± for 3 individual experiments. * $P < 0.05$ compared to basal values, + $P < 0.05$ compared to MPP+ alone; Student's *t*-test. No MPP+ (□); 500 μM MPP+ (■).

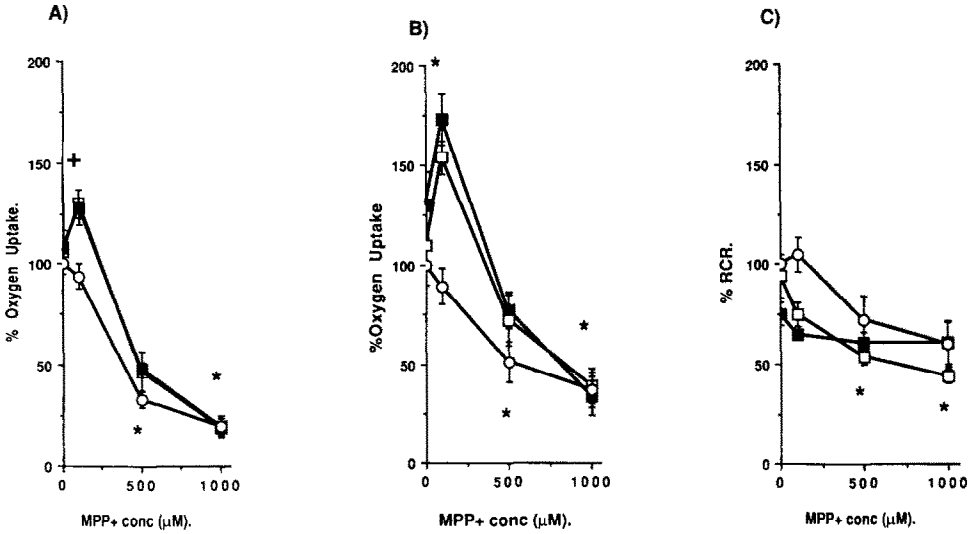

Fig. 4. The effect of SOD (50 or 100 units/mL) upon the inhibition by MPP+ (100–1000 μM) of (A) mitochondrial state 3, (B) state 4 respiration and (C) respiratory control ratio (RCR). Data are presented as the mean ± SEM for 3 individual experiments. * $P < 0.05$ compared to basal values; + $P < 0.05$ compared to equivalent MPP+ only group; Student's *t*-test. 0 units/mL SOD (○); 50 units/mL SOD (■); 100 units/mL SOD (□).

(500 and 1000 μM) the inclusion of SOD (50 or 100 units/mL) did not prevent the decrease in state 3 or 4 respiration, and the RCR values remained depressed. The addition of SOD (50 or 100 units/mL) to mitochondrial preparations containing equimolar concentrations of MPP+ and MPDP+ (100–1000 μM) did not prevent the inhibition of state 3 and 4 respiration (Fig. 5). The RCR values, although higher than those produced by MPP+/MPDP+ and MPP+ alone, were not significantly higher than basal values.

DISCUSSION

The neurotoxic action of MPTP on nigral dopamine-containing neurones and the induction of motor deficits in humans and primates is well defined, but there is controversy over the precise mechanism of action by which MPTP is toxic to dopamine-containing cells. The bioactivation of MPTP occurs via MAO B and is a prerequisite for its toxic action; MPP+ accumulates in dopamine neurones, but it is the ultimate action of MPP+ which is disputed.

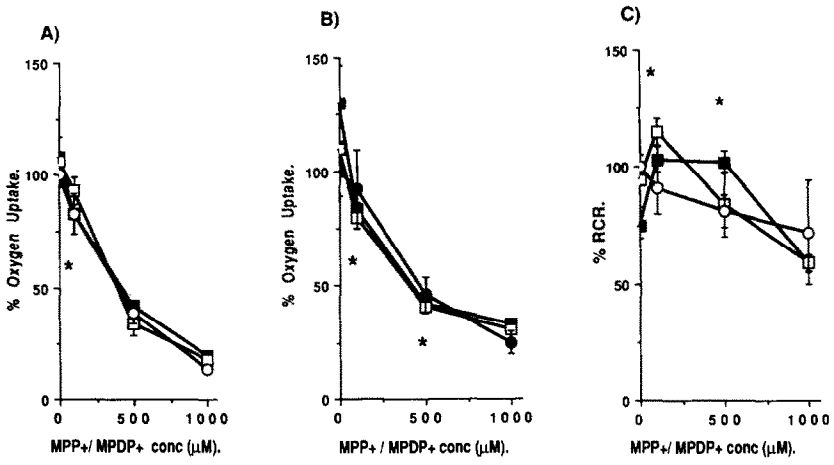

Fig. 5. The effect of SOD (50 or 100 units/mL) upon the inhibition by equimolar MPP^+ and $MPDP^+$ (100–1000 μM) of (A) mitochondrial state 3, (B) state 4 respiration and (C) respiratory control ratio (RCR). Data are presented as the mean ± SEM for 3 individual experiments. * $P < 0.05$ compared to equivalent MPP^+ + SOD group. 0 units/mL SOD (○); 50 units/mL SOD (■); 100 units/mL SOD (□).

MPP^+ can inhibit mitochondrial respiration *in vitro* [7] following active uptake into miochondria [16]. Inhibition occurs at complex I of the electron transport chain [8], between NADH dehydrogenase and the CoQ reductase. This results in depletion of ATP, a decrease in reduced glutathione levels and alterations in intracellular calcium levels. Alternatively, MPTP toxicity may result from a redox reaction between MPP^+ and $MPDP^+$ [13].

As previously demonstrated MPP^+, inhibited potently state 3 and state 4 respiration in a concentration-related manner; RCR values were also decreased. In contrast, MPTP at concentrations of up to 1000 μM did not alter mitochondrial function, in agreement with previous findings [18]. This suggests that in the mitochondrial preparations used, MPTP was not converted to MPP^+ via MAO B in amounts adequate to inhibit respiration within the incubation times used. Indeed, previous studies suggested that the enzymatic conversion of MPTP occurs only in intact cells and not in subcellular preparations [19]. The level of MAO B activity present in the mitochondrial preparations used in this study was not, apparently, high enough to produce a toxic concentration of MPP^+ from MPTP. Even at a concentration of 1000 μM MPTP, only a low concentration of $MPDP^+$ was detected and insufficient MPP^+ to be measured. Surprisingly, incorporation of $MPDP^+$ into the mitochondrial preparations did not produce inhibition of state 3 or state 4 respiration in the light or dark. Since $MPDP^+$ disproportionates to give MPTP and MPP^+ [21], some toxic effects might have been expected [22]. Indeed, focal injection of $MPDP^+$ into the substantia nigra of rats causes a depletion of dopamine in striatum [20]. The spectrophotometric analysis showed that approximately 25% of $MPDP^+$ was converted spontaneously to MPP^+. So, even at the highest concentration of $MPDP^+$ employed (1000 μM) this may have been insufficient to induce a measurable change in mitochondrial function.

Combinations of $MPDP^+$ or MPTP with MPP^+ did not increase the inhibition of mitochondrial respiration produced by MPP^+ alone. The effects of $MPDP^+$ in combination with MPP^+ were unaffected by undertaking experiments in the presence or absence of light. Previously, MPTP had been shown not to alter the inhibition of mitochondrial function produced by MPP^+ alone [8]. The present findings suggest also that if a redox reaction occurred between MPP^+ and $MPDP^+$ to produce superoxide radicals, this would not appear to influence the toxicity of MPTP metabolites found in this investigation. Further evidence for a lack of involvement of superoxide radicals came from the experiments involving superoxide dismutase. Thus, the addition of SOD did not reverse overall the inhibition of state 3 and state 4 respiration produced by MPP^+, although at higher concentrations of SOD (250–1000 μM) there was some reversal of state 3 respiration and of the inhibition of RCR. This may relate to a reversal of the effects of an accumulation of superoxide radicals induced by inhibition of complex I by MPP^+ [24]. Similarly, incorporation of SOD did not generally alter the inhibition of respiration produced by a combination of MPP^+ and $MPDP^+$. This might suggest that the proposed redox cycling mechanism between MPP^+ and $MPDP^+$ did not occur in these preparations. Alternatively, an effect of SOD on the impairment of mitochondrial respiration by MPP^+ or MPP^+ in combination may not have been observed because of the inability of SOD to penetrate into mitochondria. This study does not negate the occurrence of a redox reaction occurring between $MPDP^+$ and MPP^+ but suggests that it does not contribute to the inhibition of mitochondrial function produced by MPP^+ in brain mitochondrial preparations. The role, if any, played *in vivo* by superoxide generation from the proposed redox reaction remains to be determined.

To conclude, the results of this study support the concept of MPP^+ inhibition of mitochondrial function

as the mechanism of MPTP toxicity. The data suggest that the proposed redox reaction between the MPTP metabolites $MPDP^+$ and MPP^+ does not contribute to the effects of MPP^+ on mitochondrial respiration *in vitro*. However, the failure of $MPDP^+$ alone *in vitro* to inhibit mitochondrial respiration implies that the involvement of reactive oxygen species *in vivo* cannot be totally discounted.

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