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SHORT COMMUNICATIONS

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and free radicals *in vitro*

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Administration of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) causes degeneration of dopaminergic neurons in various animal species and induces parkinsonism in humans and primates [1–4]. MPTP is converted to $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium ion) by monoamine oxidase (MAO) B, which, in turn, disproportionates to MPTP and MPP^+ (1-methyl-4-phenylpyridinium ion) [5–7]. Deprenyl, an irreversible MAO B inhibitor, prevents the toxic effect of MPTP [8, 9]. Apparently during the biotransformation of MPTP reactive intermediates are formed which generate toxic byproducts such as superoxide and hydroxyl free radicals [10, 11]. The formation of free radical intermediates from the enzymatic reduction of MPP^+ by NADPH cytochrome P-450 reductase has been reported [12].

We detected free radical-adducts in mixtures of MPTP with mouse brain mitochondria under aerobic conditions. Free radical adducts were also detected when the metabolites of MPTP were combined in the absence of mitochondria.

Male Mice (NIH-Swiss), 20–25 g, were decapitated and crude mitochondria prepared by sucrose density gradient centrifugation from whole brain [13]. Mitochondria were suspended in 20 vol. of 50 mM phosphate buffer (pH 7.4) containing 1 mM diethylenetriaminepentaacetic acid (DETAPAC).

Reaction mixtures (0.5 ml) contained 10 mM MPTP, MPP^+ or $MPDP^+$, 90–180 mM 5,5-dimethyl-1-pyrrolidine-*N*-oxide (DMPO) and 20–100 μl (3.6–18 μg protein) of mitochondrial preparation. Immediately after the addition of MPTP, $MPDP^+$ or MPP^+, 200 μl of the mixture was transferred to a flat quartz ESR cell and placed into the cavity of a Varian Associates model E-4 ESR spectrometer. Spectra were obtained about 2–3 min after the addition of the compounds at a probe temperature of 32°. Anaerobic conditions were produced by bubbling nitrogen through the solution. Protein was measured by the procedure of Lowry *et al.* [14] with bovine serum albumin as the standard.

When mitochondria were added to DMPO, a weak signal was observed. The addition of MPTP produced the ESR spectrum shown in Fig. 1A. The signal is composed of the spectrum of the spin adduct DMPO-OH characterized by the hyperfine splitting of $A_H = A_N = 14.9$ G [15] superimposed on a six line spectrum with hyperfine coupling constants of $A_H = 22.7$ and $A_N = 15.8$ G. The origin of the latter spectrum can be attributed to the formation of a spin adduct of DMPO with a carbon-centered radical. The accumulation of the overall ESR signal increased with time, reached a maximum after approximately 20 min, and rapidly decreased. The intensity of the signal was proportional to the concentration of mitochondria added. In nitrogen-saturated mixtures no signal was detected.

Superoxide dismutase (SOD) (25 μg/ml) prevented the formation of the ESR spectrum (Fig. 1B) suggesting the generation of superoxide radicals. When the reaction mixture was preincubated for 5 min at 4° with deprenyl (1 μM), the addition of MPTP generated only a small signal (Fig. 1C). Additional evidence that inhibition of MAO B suppressed the ESR signal was provided by using a mitochondrial preparation obtained from mice pretreated with deprenyl (10 mg/kg, i.p.), 1 hr before decapitation. No ESR signal was observed in this preparation (Fig. 1D). In contrast, the addition of clorgyline (1 μM), an inhibitor of MAO A, had no appreciable effect on the ESR spectrum (data not shown).

To rule out the possibility that the inhibition of the signal was due to scavenging of free radical by the drugs rather than the inhibition of MAO, either deprenyl or clorgyline was added to solutions containing 180 mM DMPO followed by exposure for 5 min to ultrasound in a laboratory bath to generate free radicals through lysis of water molecules [16]. The ESR spectrum was not inhibited by the presence of the drugs.

To determine if the production of free radicals was related to the oxidation of MPTP and to identify the chemical steps for free radical generation, we studied solutions of $MPDP^+$ or MPP^+ in the absence of mitochondria. Under aerobic conditions, 10 mM solutions of $MPDP^+$ or MPP^+ in the presence of 90 mM DMPO produced no detectable ESR signal. However, when the solutions were mixed together, the ESR signal reported in Fig. 2A was observed. The signal increased with time, indicating continuous formation of free radicals. In the presence of SOD, the signal was strongly inhibited (Fig. 2B), suggesting that the appearance of the signal was due to the formation of superoxide radicals. No signals were generated in the absence of

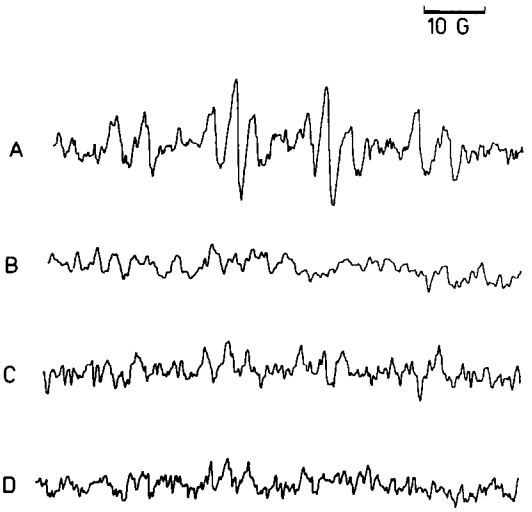

Fig. 1. ESR spectra obtained from a mixture of MPTP and a preparation of mouse brain mitochondria. The ESR cell contained 100 μl of the mitochondrial preparation, 10 mM MPTP, 90 mM DMPO in 50 mM phosphate buffer (pH 7.4) and 1 mM DETAPAC (A), 25 μg/ml of SOD (B), 1 μM deprenyl (C), and brain mitochondria prepared from mice treated with deprenyl, 10 mg/kg (D). The ESR settings were: field, 3369 G; field scan, 120 G; modulation frequency, 100 kHz; modulation amplitude, 1.6 G; nominal microwave power, 20 mW; and receiver gain, 6.3×10^3.

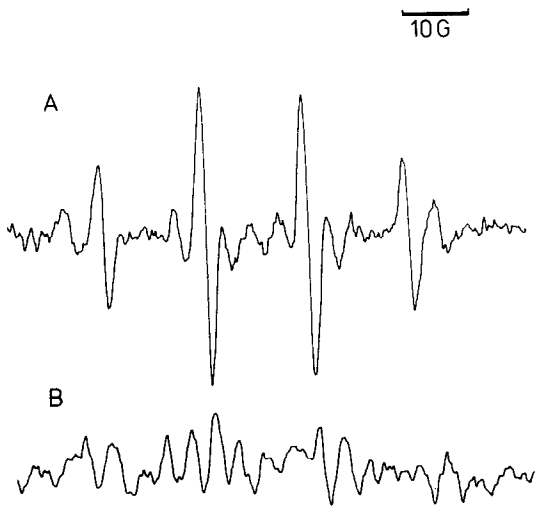

Fig. 2. ESR spectra obtained from mixtures of $MPDP^+$ and MPP^+. $MPDP^+$ and MPP^+, 10 mM each, were incubated in the presence of 90 mM DMPO in phosphate buffer containing DETAPAC, pH 7.4 (A) and in the presence of SOD, 25 μg/ml (B). See legend of Fig. 1 for other parameters.

DMPO. These experiments indicate that a redox reaction occurs between MPP^+ and $MPDP^+$, during which an electron is transferred to oxygen to form superoxide. The following reaction is suggested:

$$(a)\ MPP^+ + MPDP^+ <---> 2\,MPP\cdot + 2\,H^+$$

$$(b)\ 2\,MPP\cdot + 2\,O_2 <---> 2\,O_2^- + 2\,MPP^+$$

In summary, incubation of MPTP with mitochondria from brain resulted in oxygen-dependent formation of free radicals. Using ESR, the spin adduct of hydroxyl radical was detected. SOD suppressed the formation of radical, identifying it as superoxide. Deprenyl, an MAO B inhibitor, diminished the generation of the ESR signal, suggesting that the generation of free radicals is MAO B dependent. An ESR spectrum was obtained when the metabolites of MPTP, $MPDP^-$ and MPP^+, were incubated together in the absence of mitochondria. The formation of free radicals during the biotransformation of MPTP in brain may play a key role in its neurotoxic activity.

Departments of Pharmacology and †Chemistry
The Ohio State University
College of Medicine
Columbus, OH 43210, U.S.A.

ZVANI L. ROSSETTI*
ANTONELLO SOTGIU†
DALE E. SHARP
MARIA HADJICONSTANTINOU
NORTON H. NEFF‡

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* Visiting Associate Professor from the Institute of Pharmacology, University of Cagliari, 09100 Cagliari, Italy.
‡ Send correspondence to: Norton H. Neff, Ph.D., Department of Pharmacology, The Ohio State University, College of Medicine, 333 West Tenth Ave., 5198 Graves Hall, Columbus, OH 43210.

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Hydroxylation of aniline mediated by heme-bound oxy-radicals in a heme peptide model system

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It has been documented that heme containing proteins such as hemoglobin and myoglobin can substitute cytochrome-P450 in hydroxylation and demethylation reactions *in vitro* due to their ability to form reactive oxy-radicals during the autoxidation of their heme [1–5]. It has also been found that heme-nonapeptide (HP), a proteolytic fragment of cytochrome *c* is a good substrate of NADPH : cytochrome-P450-reductase [6]. Its inhibitory effect on lipid peroxidation in rat liver and brain microsomes [7–9] was explained by its capability of shunting electrons from microsomal reductases and/or of binding and eliminating the oxy-radicals [6].

In the widely accepted reaction scheme of drug metabolism, cytochrome-P450 is reduced by specific reductases and reoxidized by O_2. In addition, cytochrome-P450 binds and eliminates oxy-radicals [10]. Our earlier results with