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Protection and potentiation of MPTP-induced toxicity by cytochrome P-450 inhibitors and inducer: in vitro studies with brain slices

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Exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes loss of dopaminergic neurons in humans, primates and mice. Exposure of sagittal slices of mouse brain to MPTP (100 pM) caused inhibition of mitochondrial NADH-dehydrogenase activity. Leakage of lactate dehydrogenase from the slice into the medium was observed following incubation of slices with 1 nM MPTP. Neurotoxicity induced by MPTP was prevented by prior exposure of the slices to the dopamine uptake inhibitor GBR 12935. Deprenyl and pargyline (inhibitors of monoamine oxidase), also protected the slices from MPTP-induced toxicity. However, both pargyline and deprenyl also inhibited cytochrome P-450 mediated aminopyrine *N*-demethylase activity in brain slices. Pargyline, when administered in vivo to mice, decreased brain cytochrome P-450 levels significantly. Other cytochrome P-450 inhibitors, namely, piperonyl butoxide and SKF 525A were found to offer protection against MPTP induced neurotoxicity in slices without affecting monoamine oxidase activity. MPTP toxicity was potentiated significantly in brain slices prepared from mice pretreated with phenobarbital, an inducer of cytochrome P-450. The present study suggests the possible involvement of cytochrome P-450 in MPTP-induced neurotoxicity, in vitro, in brain slices.

INTRODUCTION

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a by-product of meperidine synthesis causes Parkinson's disease-like symptoms in humans[9] and primates[3]. In mouse, MPTP causes selective destruction of the dopaminergic neurons[11]. MPTP per se is not neurotoxic, but is metabolized by monoamine oxidase (MAO)[5,16] to the toxic compound *N*-methyl phenylpyridine (MPP^+), which is selectively taken up into the dopaminergic neurons, leading to cell death[18]. MPP^+ exerts its toxic effects by inhibiting mitochondrial respiration. Oxidative metabolism of NAD-linked substrates is inhibited by MPP^+ within the mitochondria, due to inhibition of NADH-dehydrogenase (NADH-DH)[19]. Prior administration of monoamine oxidase (MAO) inhibitors (namely, pargyline and deprenyl) prevents dopaminergic neurotoxicity of MPTP[12,15]. MPTP-induced toxicity is also inhibited by administration of dopamine uptake blockers[25].

Microsomal cytochrome P-450 (P-450) and flavin-containing mono-oxygenase are known to play a role in the detoxification of MPTP[4,6]. Recent reports have indicated the presence of substantial amounts of cytochrome P-450 and NADPH cytochrome P-450 reductase in brain[1,20,21,23], and their predominant localization in the mid-brain region. The present study was undertaken to examine the possible role of brain cytochrome P-450 and associated mono-oxygenase activity in MPTP-induced neurotoxicity, in vitro, using mouse brain slices.

MATERIALS AND METHODS

Male Swiss Albino mice (3 months old) from the Institute's animal house were used for the experiments. Animals had free access to pelleted diet (Hindustan Lever, Bombay, India) and water. Mice were decapitated and the brain was quickly removed and cut into sagittal slices (500 μm thickness) manually. Slices were incubated in artificial cerebrospinal fluid (CSF)[10] at 37 °C in an atmosphere of oxygen with and without MPTP. After incubation for specific time periods, slices were removed, rinsed and homogenized in 0.32 M sucrose. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant was used for the assay of NADH-DH[19]. Brain homogenate was freeze-thawed in liquid nitrogen before assay of the enzyme activity. The homogenate containing 90–120 μg of protein was added to a spectrophotometric cuvette, containing 0.28 mM NADH in phosphate buffer (0.05 M, pH 7.6) and 0.25 M sucrose. The total volume was 1 ml. Rate of change of absorbance at 340 nm was monitored at 25 °C. Leakage of cytoplasmic lactate dehydrogenase (LDH) from the slices into the medium was also measured as an indicator of cell damage[13]. After the incubation was complete, the artificial CSF was filtered through Whatman No. 1 filter paper and used for the assay of LDH. To 1 ml of phosphate buffer (50 mM, pH 7.4) containing 0.65 mM pyruvate and 11.3 mM NADH, 20 μl of artificial CSF was added and the change in

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absorbance was monitored at 340 nm.

Studies using inhibitors of P-450 and MAO were carried out by preincubating the slices at 37 °C with deprenyl, pargyline, piperonyl butoxide, SKF 525A or GBR 12935 for 30 min. MPTP (10 nM) was then added and the slices were incubated for a further period of 1 h.

Induction of mouse brain P-450 was carried out by administration of phenobarbital in drinking water (0.1%) for 10 days, followed by i.p. injections of phenobarbital (80 mg/kg b. wt.) every day for 10 days. Control animals received vehicle alone. Animals (phenobarbital-treated and control) were sacrificed 24 h after the last injection. Brain slices were prepared immediately from both groups of animals and incubated with 0.01, 0.05 and 1 nM MPTP at 37 °C for 1 h, and the slices were processed as described earlier. NADH-DH was assayed in the homogenate and LDH activity was measured in the artificial CSF. The activity of MAO was determined in the homogenate using kynuramine as substrate[14]. Brain slice homogenate was added to 1 ml of pyrophosphate buffer (pH 8.6) and 0.1 mM kynuramine, and incubated for 30 min at 37 °C. The reaction was stopped by the addition of 0.1 ml of 5% trichloroacetic acid and centrifuged. The supernatant was collected and 1 ml of sodium hydroxide (1 M) was added and the fluorescence was monitored using an excitation wavelength of 320 nm and emission wavelength 385 nm. 4-Hydroxyquinoline was used as standard. Aminopyrine demethylase activity was assayed as described earlier[20] with slight modifications. Brain slice homogenate containing 10–30 μg protein, was incubated in 0.05 M Tris buffer (pH 7.5) containing, magnesium chloride (20 mM), aminopyrine (1.8 mM), in a total volume of 1 ml. After preincubation for 3 min, the reaction was initiated by the addition of NADPH to a final concentration of 3 mM. The incubation was continued for 10 min and then the reaction was stopped by the addition of 0.5 ml of trichloroacetic acid (10% w/v). The contents were centrifuged and the formaldehyde formed was estimated in the supernatant using NASH reagent[20]. Blanks containing no NADPH were run simultaneously.

Pargyline (15 mg/kg b. wt.) was administered i.p. to mice. Animals were sacrificed 4 h later and brain microsomes were prepared from control and pargyline-treated animals and total P-450 concentration was estimated from the reduced carbon monoxide spectra[22]. Protein was estimated by the dye-binding method[2]. Data were analyzed by one-way analysis of variance and statistical significance was determined using Duncan's test. Student's *t*-test was also used where appropriate.

RESULTS

Mouse brain slices were incubated with various concentrations of MPTP at 37 °C for 1 h. Significant inhibition of NADH-DH (14%) was observed upon incubation of slices with very low concentration of MPTP (100 pM). The inhibition of NADH-DH increased with increasing concentration of MPTP (Fig. 1). When the slices were incubated with 1 mM MPTP 64% inhibition of NADH-DH was observed. Leakage of LDH from the slices into the medium was significantly increased (16%) following incubation of the slices with 1 nM MPTP (Fig. 1). Upon incubation of slices with MPTP (10 nM), significant decrease in the activity of NADH-DH was observed after 30 min of incubation, while leakage of LDH into the medium was observed after 1 h (data not shown).

Dopamine uptake inhibitor GBR 12935 effectively protected against MPTP-induced inhibition of NADH-DH (Fig. 2A) and leakage of LDH from the slice to the medium (Fig. 2A′). Preincubation of mouse brain slices with deprenyl and pargyline (MAO inhibitors), prior to exposure to MPTP (10 nM) resulted in protection against MPTP-induced inhibition of NADH-DH (Fig. 2B, C) and leakage of LDH (Fig. 2B′, C′). Slices were preincubated with P-450 inhibitors, namely, piperonyl butoxide (Fig. 2D, D′) or SKF 525A (Fig. 2E, E′) and then exposed to MPTP for 1 h, following which NADH-DH and LDH activity were measured in the slice and medium, respectively. The activities of both enzymes in incubations containing MPTP were not significantly different from controls containing inhibitors only. In control incubations containing inhibitors only, leakage of LDH from the slices was observed at higher concentration of inhibitors. Therefore, two sets of incubations were performed, one set containing various concentrations of inhibitors alone and the other set containing the inhibitors and MPTP.

The effect of the above MAO and P-450 inhibitors on

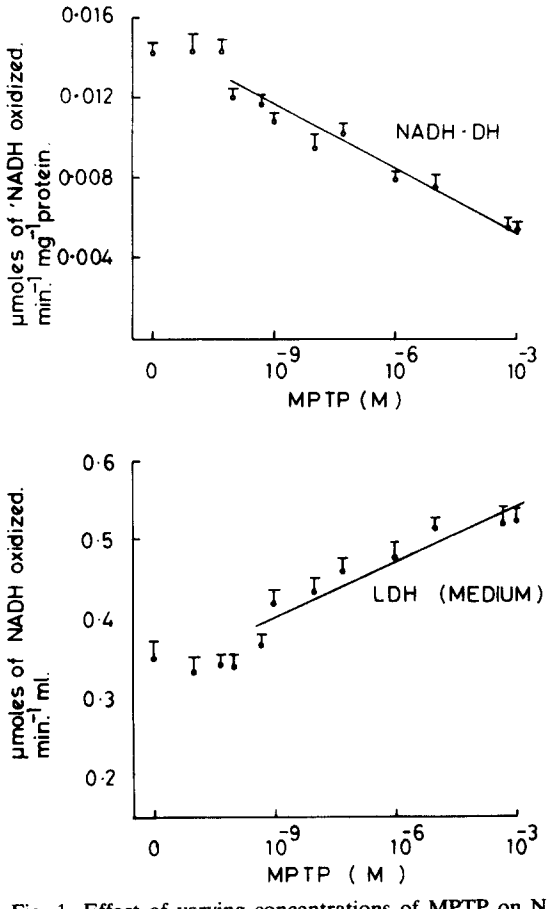

Fig. 1. Effect of varying concentrations of MPTP on NADH-DH activity and LDH leakage in mouse brain slices. Sagittal slices of mouse brain were incubated in artificial CSF containing varying concentrations of MPTP for 1 h at 37 °C. NADH-DH activity was measured in the slices, while the leakage of LDH was monitored in the medium. Values are expressed as mean ± S.E.M. (n = 6–8).

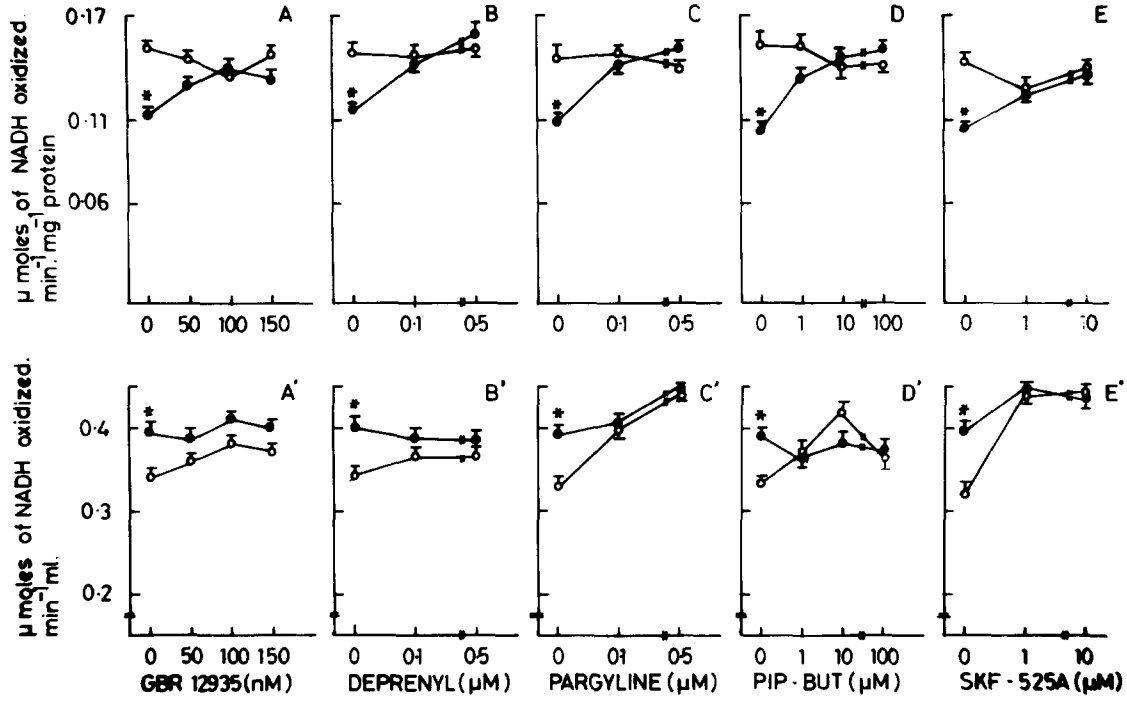

Fig. 2. Effect of inhibitors of dopamine uptake, MAO and P-450 on MPTP-induced inhibition of NADH-DH and LDH leakage in slices. Two sets of slices were preincubated for 30 min at 37 °C with varying concentrations of GBR 12935 (A, A′), deprenyl (B, B′), pargyline (C, C′), piperonyl butoxide (D, D′) and SKF 525A (E, E′), following which MPTP (10 nM) was added to one set of slices. Respective controls (○) and MPTP treated slices (●) were incubated for a further period of 1 h. The activities of NADH-DH (A–E) and LDH (A′–E′) are expressed as mean ± S.E.M. (n = 6–8). *, Values significantly different from respective controls ($P < 0.05$).

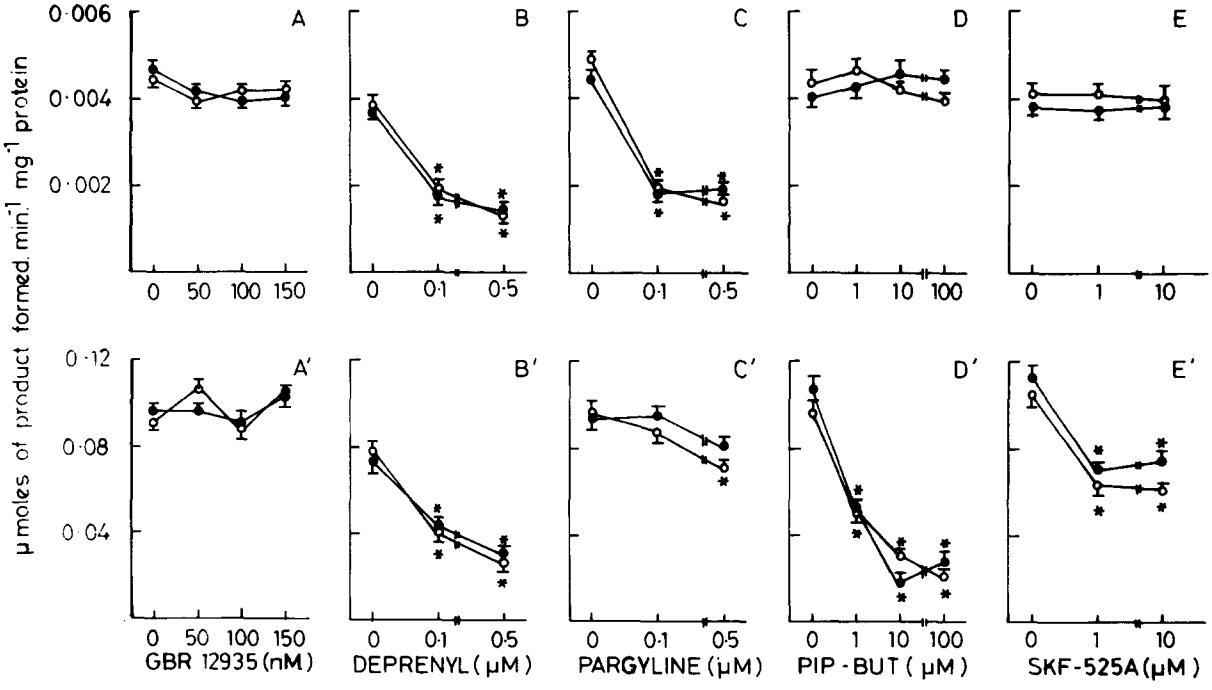

Fig. 3. The effect of various inhibitors of dopamine uptake, MAO and P-450 on MAO activity and P-450 mediated APD activity. Two sets of slices were preincubated with varying doses of GBR 12935 (A, A′), deprenyl (B, B′), pargyline (C, C′), piperonyl butoxide (D, D′) and SKF 525A (E, E′), after which MPTP (10 nM) was added to one set of slices, as indicated in Fig. 2. MAO (A–E) and P-450 mediated APD activity (A′–E′) were monitored in the slices containing inhibitor alone (○) and slices containing both inhibitor and MPTP (●). Values are mean ± S.E.M. (n = 4–6). *, Values significantly different ($P < 0.05$) from controls which did not contain any inhibitor.

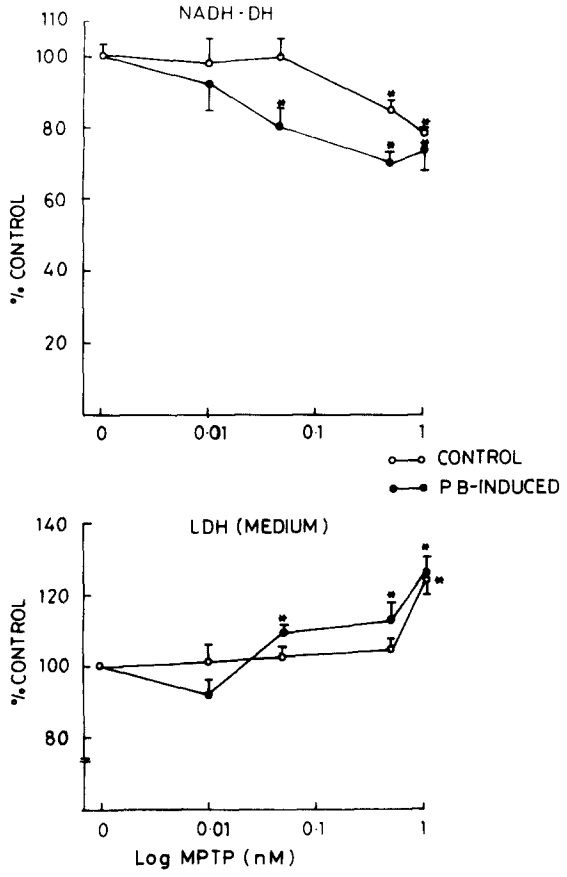

Fig. 4. Effect of phenobarbital pretreatment on MPTP-induced inhibition of NADH-DH and LDH leakage in brain slices. Brain slices were prepared from control (○) and phenobarbital-treated (●) mice and incubated with varying concentration of MPTP for 1 h at 37 °C. NADH-DH activity was measured in the slice and LDH activity was monitored in the medium. Values are expressed as mean ± S.E.M. (n = 4–6). *, Values significantly different from respective controls, which were not treated with MPTP ($P < 0.05$).

the activities of both MAO and P-450 mediated monooxygenase activity (namely, APD), in brain slices, was also investigated (Fig. 3). Both deprenyl and pargyline effectively inhibited MAO activity in brain slices in a dose-dependent manner (Fig. 3B, C), while GBR 12935 had no effect (Fig. 3A). However, pargyline and deprenyl also inhibited P-450-mediated APD activity significantly, in the slices (Fig. 3B′, C′, respectively). Piperonyl butoxide and SKF 525A, selectively inhibited APD activity (Fig. 3D, E); activity of MAO was not significantly different from controls (Fig. 3D′, E′). MPTP alone did not have any significant effect on the activity of either MAO or APD activity (Fig. 3).

In order to study the effect of pargyline in vivo, in the whole animal, pargyline (15 mg/kg) was administered i.p. to 4 mice and the animals were sacrificed 4 h later. Brain microsomes were prepared from control animals (treated with vehicle alone) and pargyline-treated animals and the total P-450 levels were measured[22]. Brain microsomal P-450 levels were decreased by 29% in pargyline treated animals. The total brain P-450 levels in control animals was 0.07 ± 0.005 nmol/mg protein, while in pargyline treated animals it was 0.05 ± 0.006 nmol/mg microsomal protein (data not shown). Pargyline also inhibited mitochondrial MAO activity by 60%. The mitochondrial MAO activity was 8.98 ± 1.0 nmol product formed/min/mg protein in control animals.

Involvement of P-450 in MPTP-induced neurotoxicity was further examined by studying the effect of induction of brain P-450 levels on MPTP-induced inhibition of NADH-DH and LDH leakage in brain slices. Brain slices were prepared from mice treated with phenobarbital and from control animals, that received vehicle alone. The slices were exposed to various concentrations of MPTP (0.01–1 nM), and the activity of NADH-DH was assayed in the slice and LDH activity was measured in the medium. Inhibition of NADH-DH and LDH leakage (Fig. 4) was observed following exposure of brain slices prepared from phenobarbital treated animals to 0.05 nM MPTP. In brain slices from control animals, similar toxicity was observed only after exposure to 1 nM MPTP. Pretreatment with phenobarbital had no effect on brain MAO activity, while P-450 mediated APD activity was induced two-fold (data not shown).

DISCUSSION

The present study demonstrates the neurotoxicity of MPTP, in vitro, in mouse brain slices at a very low concentration, namely, 0.1 nM. The concentration of MPTP in mouse brain following in vivo administration of neurotoxic dose of MPTP has been reported to be 300 μM[16]. The concentration of MPTP (500 μM) used in earlier in vitro studies has been much higher[19], than that used in the present study (0.1 nM).

The inhibition of NADH-DH by MPTP preceded the leakage of LDH from the slices (Fig. 1). Only after incubation of mouse brain slices at much higher concentration of MPTP (10 μM–1 mM), inhibition of lysosomal enzymes (*N*-acetyl glucosaminidase and acid phosphatase) and isocitrate dehydrogenase (mitochondrial enzyme) was observed (data not shown). This is in agreement with earlier studies wherein selective inhibition of NADH-DH was observed[19].

Administration of pargyline or deprenyl (MAO inhibitors) is known to protect the dopaminergic neurons from MPTP-induced damage[7,12,15]. Similar effect is seen, in vitro in the brain slices (Fig. 2). However, exposure of brain slices to pargyline or deprenyl also resulted in significant decrease of P-450 mediated APD activity (Fig. 3). As P-450 is known to exist in multiple forms in the brain, measurement of APD activity alone may not reflect the status of the total brain P-450 in the brain slice.

However, it was not possible in our hands to measure total P-450 levels in the slice, due to the small sample size. Therefore, the effect of pargyline on brain P-450 in vivo was also examined. Administration of pargyline to mice (15 mg/kg, the dose and route known to protect against MPTP toxicity[12]) also resulted in significant decrease of cerebral P-450 levels (30%). The inhibition of P-450 by deprenyl is of special interest, as it has been recommended for therapeutic use in Parkinson's disease[26]. The inhibition of the major drug metabolizing enzyme, namely, P-450 in the brain by MAO inhibitors would have a profound effect on the pharmacological modulation of other drugs given in combination with MAO inhibitors.

The effect of other well-characterised inhibitors of P-450 on MPTP-toxicity was examined. Both SKF 525A and piperonyl butoxide effectively prevented MPTP-induced damage to the slice (Fig. 2). Higher doses of piperonyl butoxide are required for the inhibition of P-450[24], as compared to the doses required for the inhibition of MAO by pargyline[12]. Therefore, the concentration of piperonyl butoxide (1 μM) used in the present study was higher than that of pargyline (0.1 μM). Both SKF 525A and piperonyl butoxide selectively inhibited P-450 mediated APD activity, but had no effect on MAO activity in the slices. Earlier studies have demonstrated that SKF 525A is not a competitive ligand for the mazindol sensitive dopamine transporter[17]. Thus, the protective effect of SKF 525A is not exerted by inhibition of MAO or inhibition of dopamine uptake.

Similar studies were carried out with MPP^+, the neurotoxic metabolite of MPTP and it was found that MPP^+ inhibited NADH-DH activity only at significantly higher concentration (10 μM, million fold higher) than the concentration of MPTP (100 pM) necessary to produce similar toxicity. Earlier in vitro studies with MPP^+ have also demonstrated that MPP^+ was toxic at very high concentrations[19]. Therefore, it was difficult to compare the relative effects of various P-450 and MAO inhibitors on MPTP and MPP^+ induced toxicity.

The protection offered by P-450 inhibitors against MPTP-induced toxicity was selective. Metarypone, a selective inhibitor of $P\text{-}450_{c,d}$[8], did not offer any protection against MPTP-mediated toxicity (data not shown). It is interesting to note that the predominant form of P-450 in the mouse brain is $P\text{-}450_{b,e}$ (which is inducible by phenobarbital); $P\text{-}450_{c,d}$ has been found to be virtually absent[20]. The potentiation of MPTP-toxicity by phenobarbital treatment thus suggests the involvement of P-450 in MPTP-induced toxicity (Fig. 4).

The present study demonstrates the possible involvement of P-450 in MPTP toxicity. However, the actual mechanism of P-450 action is yet unclear. Further, the extrapolation of the in vitro results to actual in vivo situation also needs to be determined. Regardless of the mechanism of action, inhibition of cytochrome P-450 levels in the brain may be of importance in preventing MPTP-induced damage to the brain.

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REFERENCES

1 Anandatheerthavarada, H.K., Shankar, S.K. and Ravindranath, V., Rat brain cytochrome P-450: catalytic, immunochemical properties and inducibility of multiple forms, *Brain Research,* 536 (1990) 339–343.
2 Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding, *Anal. Biochem.,* 72 (1976) 248–254.
3 Burns, R.S., Chuang, C.C., Markey, S.P., Elbert, M.H., Jacobowitz, D.M. and Kopin, I.J., A primate model for Parkinsonism: selective destruction of dopaminergic neurons in pars compacta of substantia nigra by *N*-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine, *Proc. Natl. Acad. Sci. U.S.A.,* 80 (1983) 4546–4550.
4 Cashman, J.R. and Ziegler, D.M., Contribution of N-oxygenation to the metabolism of MPTP (1-methyl-4-phenyl-1,2, 3,6-tetrahydropyridine by various liver preparations, *Mol. Pharmacol.,* 29 (1986) 163–167.
5 Chiba, K., Trevor, A. and Castognoli, N. Jr., Metabolism of neurotoxic tertiary amine, MPTP by brain monoamine oxidase, *Biochem. Biophys. Res. Commun.,* 120 (1984) 467–469.
6 Chiba, K., Kubota, E., Miyakawa, T., Kato, Y. and Ishizaki, T., Characterization of hepatic microsomal metabolism as an in vivo detoxification pathway of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice, *J. Pharmacol. Exp. Ther.,* 246 (1988) 1108–1113.
7 Cohen, C., Pasik, P., Cohen, B., Leist, A., Mytilineou, C. and Yahr, M.D., Pargyline and deprenyl prevent the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in monkeys, *Eur. J. Pharmacol.,* 106 (1984) 209–210.
8 Conney, A.H., Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons, *Cancer Res.,* 42 (1982) 4875–4917.
9 Davis, G.C. Williams, A.C., Markey, S.P., Elbert, M.H., Caine, E.D., Reichert, C.M. and Kopin, I.J., Chronic Parkinsonism is secondary to intravenous injection of meperidine analogues, *Psychiatric Res.,* 1 (1979) 249–254.
10 Elliott, K.A.C., The use of brain slices. In A. Lajtha (Ed.), *Handbook of Neurochemistry, Vol. 2,* Plenum, New York, 1969, pp. 103–114.
11 Heikkila, R.E., Hess, A. and Duvoisin, R.C., Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice, *Science,* 224 (1984) 1451–1453.
12 Heikkila, R.E., Manzino, L., Cabbat, F.S. and Duvoisin, R.C., Protection against dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by monoamine oxidase inhibitors, *Nature,* 311 (1984) 467–469.
13 Koh, J. and Choi, D.W., Zinc alters excitatory aminoacid neurotoxicity in cortical neurons, *J. Neurosci.,* 8 (1988) 2164–2171.
14 Krazl, M., A rapid microfluorimetric determination of monoamine oxidase, *Biochem. Pharmacol.,* 14 (1965) 1683–1685.
15 Langston, J.W., Irwin, J., Langston, E.B. and Forno, L.S.,

Pargyline prevents MPTP-induced Parkinsonism in primates, *Science,* 225 (1984) 1480–1482.
16 Markey, S.P., Johannessen, J.N., Chiueh, C.C., Burns, R.S. and Herkenham, M.A., Intraneuronal generation of a pyridinium metabolite may cause drug-induced Parkinsonism, *Nature,* 311 (1984) 464–467.
17 Niznik, H.B., Tyndale, R.F., Sallee, F.R., Gonzalez, F.J., Hardwick, J.P., Inaba, T. and Kalow, W., The dopamine transporter and cytochrome P-450 IIDI (Debrisoquine 4-hydroxylase) in brain: resolution and identification of 2 distinct [^{3}H] GBR 12935 binding proteins, *Arch. Biochem. Biophys.,* 276 (1990) 424–432.
18 Ramsay, R.R. and Singer, T.P., Energy-dependent uptake of *N*-methyl-4-phenyl pyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, by mitochondria, *J. Biol. Chem.,* 261 (1986) 7585–7587.
19 Ramsay, R.R., Salach, J.I., Dadgar, J. and Singer, T.P., Inhibition of mitochondrial NADH dehydrogenase by pyridine derivatives and its possible relation to experimental and idiopathic Parkinsonism, *Biochem. Biophys. Res. Commun.,* 135 (1986) 259–275.
20 Ravindranath, V. and Anandatheerthavarada, H.K., High activity of cytochrome P-450 linked aminopyrine *N*-demethylase in mouse brain microsomes and associated sex-related difference, *Biochem. J.,* 261 (1989) 769–773.
21 Ravindranath, V., Anandatheerthavarada, H.K. and Shankar, S.K., NADPH cytochrome P-450 reductase in rat, mouse and human brain, *Biochem. Pharmacol.,* 39 (1990) 1013–1018.
22 Ravindranath, V. and Anandatheerthavarada, H.K., Preparation of brain microsomes with cytochrome P-450 activity using calcium aggregation method, *Anal. Biochem.,* 187 (1990) 310–313.
23 Ravindranath, V., Anandatheerthavarada, H.K. and Shankar, S.K., Xenobiotic metabolism in human brain. Presence of cytochrome P-450 and associated mono-oxygenase, *Brain Research,* 496 (1989) 331–335.
24 Ravindranath, V., McMenamin, M.G., Dees, J.H. and Boyd, M.R., Hepatotoxicity of 2-methylfuran — role of metabolic activation in vivo, *Toxicol. Appl. Pharmacol.,* 85 (1986) 78–91.
25 Sundestrom, E. and Jonnson, G., Pharmacological interference with the neurotoxic action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on central catecholaminergic neurons in the mouse, *Eur. J. Pharmacol.,* 110 (1985) 293–299.
26 The Parkinson Study Group, Effect of deprenyl on the progression of disability in early Parkinson's disease, *N. Engl. J. Med.,* 321 (1989) 1364–1370.