ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 255, No. 1, May 15, pp. 14-18, 1987

Comparative Toxicity and Antioxidant Activity of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine and Its Monoamine Oxidase B-Generated Metabolites in Isolated Hepatocytes and Liver Microsomes[1]

GUNILLA EKSTRÖM,[2] DONATO DI MONTE, MARTHA S. SANDY, AND MARTYN T. SMITH[3]

Department of Biomedical and Environmental Health Sciences, School of Public Health, University of California, Berkeley, California 94720

Received October 16, 1986

MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is converted by monoamine oxidase B to its putative toxic metabolite MPP^+ (1-methyl-4-phenylpyridinium ion) via $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium ion). Both the parent compound and these two major metabolites were toxic to isolated rat hepatocytes with $MPDP^+$ being the most toxic and MPP^+ the least effective. MPP^+ produced a slight increase in lipid peroxidation above control levels in hepatocytes, while both MPTP and $MPDP^+$ showed antioxidant effects. The latter two compounds also protected against chemically and nonchemically induced lipid peroxidation in rat liver microsomes. $MPDP^+$ was effective at much lower concentrations than MPTP. $MPDP^+$ was also markedly more efficient when NADPH was used to induce microsomal lipid peroxidation. Lipid peroxidation as a consequence of oxygen radical generation is therefore unlikely to be involved in MPTP toxicity *in vitro* and the rationale of using chain-breaking antioxidants as protective agents *in vivo* needs a more careful evaluation.

The neurotoxic effects of MPTP[4] (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), which ultimately lead to a clinical picture of parkinsonism in humans and other primates (1), require its metabolic activation by monoamine oxidase B (MAO-B) (2). This oxidative pathway produces the putative toxic metabolite MPP^+ (1-methyl-4-phenylpyridinium ion) via a dihydropyridinium intermediate $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium ion) (3). The toxic effects of MPP^+ have been attributed to the generation of active oxygen species and the subsequent oxidative destruction of cell membrane lipids (4, 5). Attempts to prevent the toxic consequences of MPTP exposure *in vivo* via the use of antioxidants have led, however, to controversial results (6, 7). Furthermore, in recent work with isolated rat hepatocytes, we have dissociated the cytotoxic effects of MPP^+ and MPTP from the production of oxygen radicals (8, 9).

In this study we extend our investigations to $MPDP^+$. The role played by this unstable metabolite in MPTP toxicity is still relatively unknown despite the fact

[1] This work was supported by the Odd Fellows Lodge, Stockholm, Sweden (G.E.), the Health Effects Component of University of California Toxic Substances Program (D.D.), the Northern California Occupational Health Center (M.S.S.), and the National Foundation for Cancer Research (M.T.S.).

[2] Present address: Department of Physiological Chemistry, Karolinska Institute, 104 01 Stockholm, Sweden.

[3] To whom all correspondence should be addressed.

[4] Abbreviations used: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MAO-B, monoamine oxidase B; MPP^+, 1-methyl-4-phenylpyridinium ion; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium ion; TCA, trichloroacetic acid; TBA, thiobarbituric acid; 1,2-MPDP, 1-methyl-4-phenyl-1,2-dihydropyridine.

0003-9861/87 $3.00

that it has been shown to produce damage to dopaminergic neurons in the substantia nigra (10). Furthermore, we relate the cytotoxicity of MPTP and its MAO-B-generated metabolites to their effects on lipid peroxidation, in order to test the rationale of using antioxidants as protective agents.

MATERIALS AND METHODS

Hepatocytes were isolated from male Sprague-Dawley rats (180-250 g) and incubated at 37°C in Krebs-Henseleit buffer, pH 7.4 (11), under a 95% O_2/5% CO_2 atmosphere. Cell viability was assessed by trypan blue exclusion (11). Liver microsomes were prepared from similar male Sprague-Dawley rats as described in Ref. (12). Protein was determined as described by Lowry *et al.* (13). Reaction mixtures contained microsomes corresponding to 0.5 mg of protein in 50 mM potassium phosphate buffer, pH 7.4, in a final volume of 1 ml. The incubations were started by the addition of 100 μl of 500 μM cumene hydroperoxide or 50 μl of 4.8 mM NADPH and terminated by the addition of 0.25 ml 40% trichloroacetic acid (TCA) and 0.125 ml 5 M HCl. Control incubations were carried out by adding cumene hydroperoxide or NADPH after the addition of TCA. The uv irradiation was performed at 254 nm at a distance of 4 cm for 10 min. The efficiency of the lamp was 580 W/cm^2 at 15 cm. Microsomes were diluted to 0.5 mg/ml with 50 mM potassium phosphate buffer, pH 7.4, in small petri dishes. MPTP and MPDP$^+$ were added just prior to irradiation. Lipid peroxidation products were measured using the thiobarbituric acid (TBA) assay (14). Cytochrome *c* reduction was measured spectrophotometrically at 550 nm. MPTP, MPDP$^+$, and MPP$^+$ were kindly supplied by Professor Neal Castagnoli, Jr., Division of Toxicology, Department of Pharmacy, University of California, San Francisco.

RESULTS AND DISCUSSION

When isolated rat hepatocytes were exposed to equimolar concentrations of the parent compound MPTP or either of the two major metabolites (MPDP$^+$, MPP$^+$), results as in Fig. 1A were obtained. The loss of cell viability ultimately occurred in 100% of hepatocytes after addition of any of the three compounds, but was most rapid after MPDP$^+$ exposure, and occurred only after a relatively long lag period in the presence of MPP$^+$. Due to their charged structures, both MPDP$^+$ and MPP$^+$ could be expected to have limited accessibility to hepatocytes. MPDP$^+$, however, was the most toxic of the three compounds studied and is likely to be readily taken up by hepatocytes, since rapid intracellular biochemical changes followed its addition to the incubation mixtures (data not shown). The passive diffusion across cell membranes of the lipophilic free base 1,2-MPDP (1-methyl-4-phenyl-1,2-dihydropyridine) (Fig. 2) might account for the ready accessibility of the corresponding charged acid (MPDP$^+$) to the cell (15).

It has been suggested that a free radical-mediated mechanism could be involved in MPTP toxicity (4, 5) and antioxidants have been proposed as protective agents against oxygen radical-induced lipid peroxidation after MPTP exposure (7). We therefore measured lipid peroxidation products in hepatocyte incubations after the addition of 1.5 mM MPTP, MPDP$^+$, or MPP$^+$ (Fig. 1B). A slightly increased formation of these

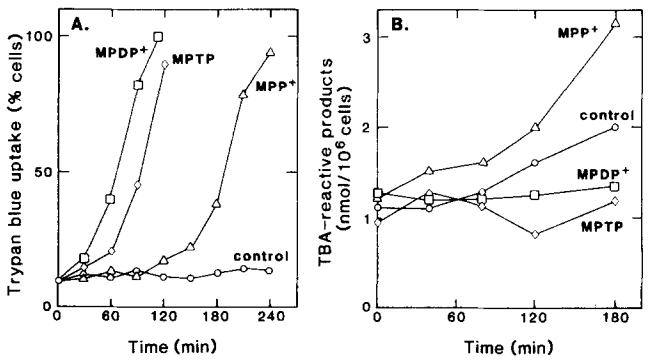

FIG. 1. Cell viability (A) and lipid peroxidation (B) after exposure of isolated hepatocytes to 1.5 mM MPTP (◇), 1.5 mM MPDP$^+$ (□), 1.5 mM MPP$^+$ (△), or no addition (○).

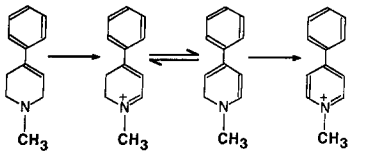

FIG. 2. Structures of MPTP and its major metabolites: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium ion; 1,2-MPDP, 1-methyl-4-phenyl-1,2-dihydropyridine; MPP^+, 1-methyl-4-phenylpyridinium ion.

products, as compared to control cells, was observed only in the presence of MPP^+, the least toxic of the three pyridine compounds. On the other hand, exposure to both MPTP and $MPDP^+$ produced rates of lipid peroxidation even lower than those measured in untreated cells. We decided to further investigate these antioxidant properties of MPTP and $MPDP^+$ in a noncellular system, namely rat liver microsomes.

Organic hydroperoxides can initiate microsomal lipid peroxidation by the cytochrome *P*-450-dependent formation of peroxy and alkoxy radicals (16). The generation of TBA-reactive products following a 5-min incubation of microsomes with 50 μM cumene hydroperoxide was inhibited by 50% in the presence of 1 mM MPTP and by 75% in the presence of 0.5 mM $MPDP^+$ (Fig. 3). No effect was observed after the addition of 1 mM MPP^+ (not shown). Lipid peroxidation can also be induced nonchemically by uv irradiation. The presence of either MPTP or $MPDP^+$ during irradiation protected microsomes against peroxidative damage (Fig. 4), which shows that these compounds are direct chemical antioxidants. $MPDP^+$ was more effective in preventing this lipid peroxidation than MPTP, producing an 80% inhibition at concentrations 10 times lower than those of MPTP.

When NADPH was added to microsomal incubations to induce lipid peroxidation via NADPH-cytochrome *P*-450 (*c*) reductase (17), $MPDP^+$ was even more efficient at inhibiting the formation of TBA-reactive products. It was able to protect significantly at concentrations as low as 0.05 mM, 100 times lower than with MPTP (Fig. 5). $MPDP^+$ also decreased the rate of NADPH-dependent cytochrome *c* reduction in liver microsomes (Fig. 6). This effect, however, was significant only at concentrations much higher than 0.05 mM, indicating that the protective effect of $MPDP^+$ against NADPH-induced lipid peroxidation cannot simply be a consequence of inhibition of the activity of NADPH-cytochrome *P*-450 (*c*) reductase. A more probable antioxidant mechanism is that $MPDP^+$, and to a lesser extent MPTP, reacts directly with the initiating species, a proposed superoxide radical/iron complex (18).

In conclusion, both MPTP and $MPDP^+$ do not induce, but indeed inhibit lipid peroxidation in rat hepatocytes and liver mi-

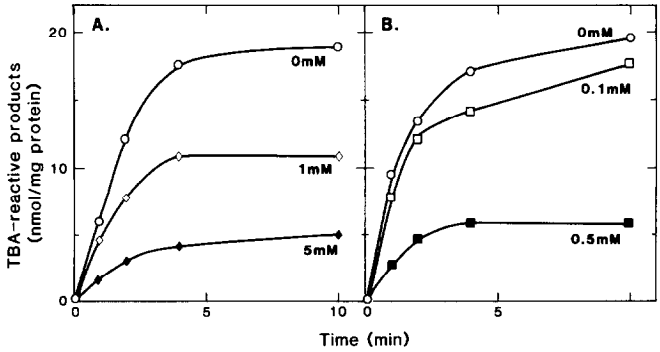

FIG. 3. Effect of MPTP (A) and $MPDP^+$ (B) on liver microsomal lipid peroxidation initiated by 50 μM cumene hydroperoxide. Reaction mixtures contained microsomes corresponding to 0.5 mg of protein alone (○) and with 1 mM MPTP (◇), 5 mM MPTP (◆), 0.1 mM $MPDP^+$ (□), or 0.5 mM $MPDP^+$ (■). Incubations were performed at 30°C. Results shown are the means of four separate incubations.

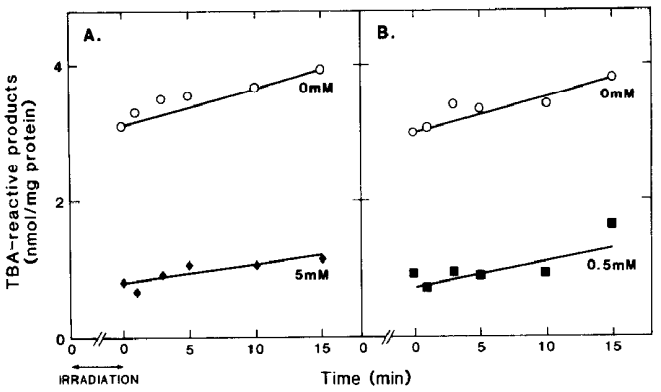

FIG. 4. Protective effect of MPTP (A) and $MPDP^+$ (B) against formation of TBA-reactive products induced by uv irradiation. Samples were taken at indicated time points (following 10 min of irradiation) from petri dishes containing microsomes (0.5 mg of protein/ml) alone (○), with 5 mM MPTP (♦), or with 0.5 mM $MPDP^+$ (■). The amount of TBA-reactive products in the samples prior to irradiation has been subtracted. Results shown are the means of four separate experiments.

crosomes. This effect in the presence of MPTP does not seem to be related to its metabolism. Pargyline, an inhibitor of MAO-B, did not modify the antioxidant effects of MPTP in isolated hepatocytes (data not shown). Furthermore, MPTP was able to protect against lipid peroxidation in microsomes even in the absence of NADPH, when no evidence of metabolite formation has been reported (19). The results reported here lead to the conclusion that lipid peroxidation is unlikely to be involved in the general cytotoxic effects of MPTP. Whether or not lipid peroxidation plays any role in the selective toxicity of this compound to

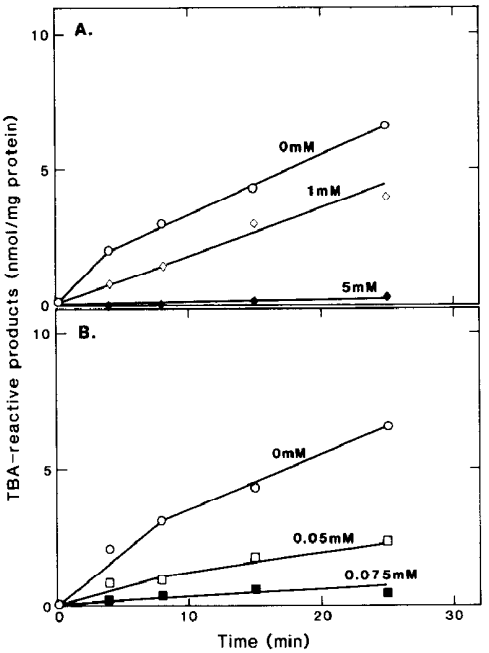

FIG. 5. Inhibitory effects of MPTP (A) and $MPDP^+$ (B) on NADPH-dependent microsomal lipid peroxidation. Reaction mixtures (1 ml) consisted of microsomes corresponding to 0.5 mg of protein and 0.2 mg NADPH (○) with 1 mM MPTP (◇), 5 mM MPTP (♦), 0.05 mM $MPDP^+$ (□), or 0.075 mM $MPDP^+$ (■). Incubations were performed at 30°C. Results shown are the means of three separate incubations.

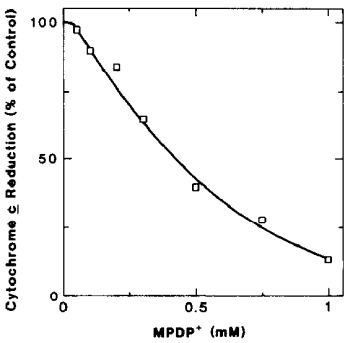

FIG. 6. Effect of $MPDP^+$ on NADPH-dependent cytochrome *c* reduction in liver microsomes. Rat liver microsomes (0.5 mg/ml) were incubated at 30°C in 50 mM potassium phosphate buffer (pH 7.6) in the presence of cytochrome *c* (0.6 mg/ml) and NADPH (20 μg/ml). Cytochrome *c* reduction was monitored at 550 nm following the addition of increasing concentrations of $MPDP^+$. Data represent the means of duplicate experiments.

the dopaminergic neurons of the substantia nigra remains an open question, however. An alternative mechanism of cytotoxicity involving accumulation of MPP^+ in mitochondria (20), the subsequent inhibition of mitochondrial respiration (9, 21), and disruption of calcium homeostasis (22) seems more likely. The latter has been shown to be important in the cytotoxicity of numerous compounds to hepatocytes (23) and may well be important in the neuronal toxicity of MPTP. Why nigrostriatal neurons should be so sensitive to the toxic effects of MPTP and/or its metabolites is a subject for further study. The fact that both MPTP and $MPDP^+$ are quite potent antioxidants in their own right cannot be ignored, however, and the rationale of using antioxidants as protective agents against parkinsonism induced by MPTP or "MPTP-like" substances needs careful evaluation.

ACKNOWLEDGMENTS

We thank Drs. Neal Castagnoli, Jr., and Anthony Trevor for much helpful discussion.

REFERENCES

1. LANGSTON, J. W., BALLARD, P., TETRUD, J. W., AND IRWIN, I. (1983) *Science* **219,** 979-980.
2. HEIKKILA, R. E., MANZINO, L., CABBAT, F. S., AND DUVOISIN, R. C. (1984) *Nature (London)* **311,** 467-469.
3. CHIBA, K., TREVOR, A. J., AND CASTAGNOLI, N., JR. (1984) *Biochem. Biophys. Res. Commun.* **120,** 547-578.
4. MARKEY, S. P., JOHANESSEN, J. N., CHIUEH, C. C., BURNS, R. S., AND HERKENHAM, M. A. (1984) *Nature (London)* **311,** 464-467.
5. SINHA, B. K., SINGH, Y., AND KRISHNA, G. (1986) *Biochem. Biophys. Res. Commun.* **135,** 583-588.
6. BALDESSARINI, R. J., KULA, N. S., FRANCOEUR, D., AND FINKLESTEIN, S. P. (1986) *Neurology* **36,** 737.
7. WAGNER, G. C., JARVIS, M. F., AND CARELLI, R. M. (1985) *Neuropharmacology* **24,** 1261-1262.
8. DI MONTE, D., SANDY, M. S., EKSTRÖM, G., AND SMITH, M. T. (1986) *Biochem. Biophys. Res. Commun.* **137,** 303-309.
9. DI MONTE, D., JEWELL, S. A., EKSTRÖM, G., SANDY, M. S., AND SMITH, M. T. (1986) *Biochem. Biophys. Res. Commun.* **137,** 310-315.
10. JONSSON, G., SUNDSTRÖM, E., NWANZE, E., HALLMAN, H. AND LUTHMAN, J. (1986) *in* MPTP: A Neurotoxin Producing a Parkinsonism Syndrome (Markey, S. P., Castagnoli, N., Jr., Trevor, A. J., and Kopin, I. J., Eds.), pp. 253-272, Academic Press, New York.
11. MOLDEUS, P., HÖGBERG, J., AND ORRENIUS, S. (1978) *in* Methods in Enzymology (Fleischer, S., and Packer, L., Eds.), Vol. 52, pp. 60-71, Academic Press, New York.
12. ERNSTER, L., SIKEVITZ, P., AND PALADE, G. E. (1962) *J. Cell Biol.* **15,** 541-562.
13. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265-275.
14. BERNHEIM, F., BERNHEIM, M. L., AND WILBER, K. M. (1948) *J. Biol. Chem.* **174,** 257-264.
15. CHIBA, K., TREVOR, A. J., AND CASTAGNOLI, N., JR. (1986) *in* MPTP: A Neurotoxin Producing a Parkinsonism Syndrome (Markey, S. P., Castagnoli, N., Jr., Trevor, A. J., and Kopin, I. J., Eds.), pp. 575-579, Academic Press, New York.
16. CADENAS, E., AND SIES, H. (1982) *Eur. J. Biochem.* **124,** 349-356.
17. HOCHSTEIN, P., NORDENBRAND, K., AND ERNSTER, L. (1964) *Biochem. Biophys. Res. Commun.* **14,** 323-328.
18. PEDERSON, T. C., AND AUST, S. D. (1972) *Biochem. Biophys. Res. Commun.* **48,** 789-796.
19. WEISSMAN, J., TREVOR, A. J., CHIBA, K., PETERSON, L. A., CALDERA, P., AND CASTAGNOLI, N., JR. (1985) *J. Med. Chem.* **28,** 997-1001.
20. RAMSAY, R. R., SALACH, J. I., AND SINGER, T. P. (1986) *Biochem. Biophys. Res. Commun.* **134,** 743-748.
21. NICKLAS, W. J., VYAS, I., AND HEIKKILA, R. E. (1985) *Life Sci.* **36,** 2503-2508.
22. FREI, B., AND RICHTER, C. (1986) *FEBS Lett.* **198,** 99-102.
23. JEWELL, S. A., BELLOMO, G., THOR, H., ORRENIUS, S., AND SMITH, M. T. (1982) *Science* **217,** 1257-1259.