The Content of Intracellular Mitochondrial DNA Is Decreased by 1-Methyl-4-phenylpyridinium Ion (MPP⁺)*

(Received for publication, December 27, 1996, and in revised form, February 12, 1997)

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1-Methyl-4-phenylpyridinium ion (MPP⁺), an oxidative metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is considered to be directly responsible for MPTP-induced Parkinson's disease-like symptoms by inhibiting NADH-ubiquinone oxidoreductase (complex I) in the mitochondrial respiratory chain. Here we demonstrate that 25 μM MPP⁺ decreases the content of mitochondrial DNA to about one-third in HeLa S3 cells. On the contrary, 0.1 μM rotenone, which inhibits complex I to the same extent as 25 μM MPP⁺ in the cells, increases the content of mitochondrial DNA about 2-fold. Hence, the effect of MPP⁺ on mitochondrial DNA is not mediated by the inhibition of complex I. To examine the replication state of mitochondrial DNA, we measured the amount of nascent strands of mitochondrial DNA. The amount is decreased by MPP⁺ but increased by rotenone, suggesting that the replication of mitochondrial DNA is inhibited by MPP⁺. Because the proper amount of mitochondrial DNA is essential to maintain components of the respiratory chain, the decrease of mitochondrial DNA may play a role in the progression of MPTP-induced Parkinson's disease-like symptoms caused by the mitochondrial respiratory failure.

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Parkinson's disease is a common neurodegenerative disease with motor abnormalities resulting from the selective dopaminergic cell death in the substantia nigra pars compacta. Since 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was found to cause Parkinson's disease in human, it is widely used to produce an experimental model of Parkinson's disease in primates, rats, and mice (1–3). Animals that receive MPTP show a marked reduction in the number of dopaminergic cells in the substantia nigra pars compacta. MPTP is oxidized to 1-methyl-4-phenylpyridinium ion (MPP⁺). MPP⁺ is selectively accumulated in the dopaminergic cells via dopamine transporter and causes the dopaminergic cell death. MPP⁺, like rotenone, is an inhibitor of NADH-ubiquinone oxidoreductase (complex I) in the mitochondrial respiratory chain (4–6). The inhibition of complex I not only leads to the decline of the mitochondrial ATP production, but also causes the generation of superoxide radical (O₂⁻) and induces lipid peroxidation reaction (7–9). It has been demonstrated that MPP⁺ stimulates the production of O₂⁻ and initiates lipid peroxidation reaction in isolated bovine heart mitochondria (10). The oxygen radicals as well as intracellular ATP depletion are implicated in the MPP⁺-induced cell death. Consistent with this assumption, transgenic mice overexpressing the copper/zinc-type superoxide dismutase (11) and mutant mice lacking neuronal nitric-oxide synthase (12) are both resistant to MPTP-induced dopamine neurotoxicity. Thus, MPP⁺ apparently functions as an agent of oxidative stress to mitochondria in vivo.

Thirteen subunits of the respiratory chain are coded by mitochondrial DNA, all of which are essential to the respiratory electron transport system. The integrity of mitochondrial genome must be maintained for the normal respiratory function. Mitochondria have the machinery for preventing the mutations caused by oxidative damages (13, 14). The adequate amount of transcripts from the genome is critical for maintaining mitochondrial function in addition to preventing mutations. Particularly on the level of transcripts from the mitochondrial genome, we should take the copy number of DNA into account as well as the efficiency of the initiation of transcription from each genome, since mitochondrial DNA exists in multicopies. The decline of the copy number of mitochondrial DNA should lead to the decrease in the transcript level. The replication of mitochondrial DNA must be regulated precisely for maintaining the copy number. The replication of mitochondrial DNA is tightly coupled to transcription (15). The initiation of transcription of mitochondrial DNA is mainly regulated in the D-loop region where the cis-acting elements are almost exclusively located. The D-loop region is considered to be more susceptible to oxidative stress than the other regions (16). Hence, we expected that oxidative damages to mitochondrial DNA could alter the degree of the initiation of transcription and replication.

Despite the essential role of mitochondrial DNA in respiration, the effects of MPP⁺ on mitochondrial DNA have not been adequately investigated. Considering the possibility that MPP⁺ may affect the integrity of mitochondrial DNA through oxidative damage, we examined the in vivo effect of MPP⁺ on the mitochondrial genome. Here, we demonstrate that MPP⁺ decreases the content of mitochondrial DNA without apparent degradation of the DNA, and the decrease is likely due to the selective inhibition of the replication of mitochondrial DNA.

**Experimental Procedures**

Materials—BamHI, XbaI, PvuII, and T4 DNA ligase were purchased from Takara (Seta, Japan). RNase A and rotenone were from Sigma. Vent DNA polymerase was from New England Biolabs (Beverly, MA). MPP⁺ was from Research Biochemicals International (Natick, MA). EDTA was from Dojindo (Kumamoto, Japan). Other reagents were of analytical grade.

Cell Culture—HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 10 units/liter penicillin G, and 100 μg/ml streptomycin. The cell number was determined photometrically using WST-1 cell counting kit (Dojindo).

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*This work was supported in part by Grant-in-aid for Scientific Research on Priority Areas (08280104) from the Ministry of Education, Science, Sports, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP⁺, 1-methyl-4-phenylpyridinium ion; PCR, polymerase chain reaction; LMPCR, ligation-mediated PCR; O₂⁻, superoxide radical; kb, kilobase pair(s); nt, nucleotide(s).
MPP production of ATP in the cells. We first examined the effect of extent to which mitochondrial respiration contributes to the cated concentration of MPP under "Experimental Procedures." B, after incubation with the indicated concentration of MPP for 3 days, the content of mitochondrial DNA (Mt DNA) in 0.5 μg of the PvuII-digested total DNA was estimated by Southern blot using the 0.8-kbp XhoI fragment of mitochondrial DNA (nt 7441–8286) as a probe. The 18 S rRNA gene was also detected simultaneously as an internal standard for the nuclear gene.

Southern Blot—The cells cultured in the indicated conditions were harvested and the total DNA was extracted with DNAzol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. The extracted DNA was digested with RNase A and PvuII, phenol-extracted, ethanol-precipitated, and solubilized in 0.1 × TE (1 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA). The concentration of DNA was determined by measuring A_{260}. After 0.5 μg of DNA was electrophoresed in 0.6% agarose gel and transferred onto nylon membrane (Hybond-N, Amersham Corp., Buckinghamshire, United Kingdom), the mitochondrial gene and 18 S rRNA gene were probed with 0.8-kbp XhoI fragment of mitochondrial DNA (nt 7441–8286) and 1.5-kbp XhoI fragment of mitochondrial DNA, respectively. Each probe was labeled with XbaI fragment of mitochondrial DNA (nt 7441–8286) and 1.5-kbp XhoI fragment of mitochondrial DNA. The 18 S rRNA gene was also detected simultaneously as an internal standard for the nuclear gene.

Ligation-mediated PCR—Ligation-mediated PCR (LMPCR) of the mitochondrial D-loop region was performed as reported previously (17)2 and imaged with FluorImagerSI (Molecular Dynamics). Primers 1 and 2 were 5'-tttcgtctggggggtatgc-3' and 5'-cagcgagagacctctc-3', respectively. Primer 2 was fluorescein labeled at the 5' end.

Lactate Production—The production of lactate was estimated by the increase of lactate in the culture medium. Lactate was measured as described previously (19).

RESULTS AND DISCUSSION

Effects of MPP+ on Cell Growth and DNA Content—Effect of MPP+ on cell viability varies in cell lines depending on the extent to which mitochondrial respiration contributes to the production of ATP in the cells. We first examined the effect of MPP+ on the growth of HeLa S3 cells. MPP+ dose-dependently inhibited the growth. The growth was inhibited by about 30% at 25 μM MPP+ (Fig. 1A). The intensity of 16-kbp band of mitochondrial DNA decreased to about one-third at 25 μM MPP+ (Fig. 1A). From the fact that we did not observe the increase of smear bands under the 16-kbp band, the decrease of mitochondrial DNA was apparently not due to its degradation.

Effects of Rotenone on Cell Growth and DNA Content—To determine whether the decrease of mitochondrial DNA by MPP+ is mediated by the inhibition of complex I, we examined the effects of rotenone on cell growth and the content of mitochondrial DNA. MPP+ is considered to bind to the rotenone-binding site in complex I, thereby inhibiting complex I (6). Rotenone inhibited the growth of HeLa S3 cells dose-dependently (Fig. 2A). Just like 25 μM MPP+, 0.1 μM rotenone inhibited the growth by approximately 30%.

To examine to what extent 0.1 μM rotenone and 25 μM MPP+ inhibits complex I in the HeLa cells, we measured the production of lactate by the cells treated with each reagent, because the inhibition of complex I decreases the content of intracellular ATP, resulting in the compensating enhancement of the glycolytic activity. As shown in Table I, both reagents enhanced the production of lactate to a similar extent, suggesting that the two reagents inhibit complex I similarly in vivo.

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* D. Kang, K. Miyako, Y. Kai, T. Irie, and K. Takeshige, submitted for publication.
TABLE I
Lactate production
HeLa S3 cells were cultured with MPP⁺ or rotenone at the indicated concentrations for 72 h. The content of lactate in the medium was measured. The difference in the content of lactate before and after culture was calculated and represents the production of lactate by the cells. The values are a mean of two independent experiments. Each value in parentheses represents the relative increase of the lactate production.

| MPP⁺  | µmol/10^⁶ cells |
|-------|-----------------|
| 0 µM  | 2.8 (1)         |
| 25 µM | 7.4 (2.6)       |
| Rotenone⁺ | 3.3 (1) |
| 0.1 µM | 8.6 (2.6)       |

* 0.1% ethanol is contained in the culture medium.

In contrast to 25 µM MPP⁺, the intensity of mitochondrial DNA band increased approximately 2-fold at 0.1 µM rotenone concentration, when the mitochondrial DNA was detected in the BamHI-digested total DNA with the 1.9-kbp fragment of mitochondrial DNA (nt 2573–4431) (Fig. 2B). To confirm the increase in mitochondrial DNA content, we probed mitochondrial DNA and 18 S rRNA gene simultaneously in the PvuII-digested total DNA. We obtained essentially the same results as in the BamHI-digested total DNA (Fig. 2C). These results show that the inhibition of complex I alone does not decrease the content of mitochondrial DNA.

Effect on the Content of Nascent Strand of Mitochondrial DNA—Apparently the decrease of mitochondrial DNA was not accompanied with the degradation (Fig. 1A). This prompted us to examine the replication of mitochondrial DNA. We developed the ligation-mediated PCR method to estimate the content of the nascent strands of mitochondrial DNA.² We specifically amplified the strands with the free 5' end, i.e. nascent strands of mitochondrial DNA from the cells treated with or without MPP⁺. To assess the number of the nascent strand per mitochondria, we adjusted the amount of mitochondrial DNA in between MPP⁺-treated and MPP⁺-untreated cells in the LMPCR analysis based on the quantification of mitochondrial DNA by Southern blot. MPP⁺ weakened signals of free 5' ends (Fig. 3), which indicates that the number of nascent strand per mitochondrial DNA decreased. On the other hand, rotenone augmented the signals (Fig. 3). These results suggest that MPP⁺ inhibits the replication of mitochondrial DNA, while rotenone enhances it.

Although rotenone has not been reported to increase the intracellular content of mitochondrial DNA or enhance the replication to date, it is plausible that the content of mitochondrial DNA compensatingly increases when the respiratory function declines due to the inhibition of complex I as shown here. We noted that the content of mitochondrial DNA in the cells treated with 50 µM MPP⁺ was slightly more than that found in the cells with 25 µM MPP⁺ reproducibly (Fig. 1B). This increase of mitochondrial DNA may result from the fact that the mitochondrial DNA-decreasing effect of MPP⁺ was partially compensated by the mitochondrial DNA-increasing effect caused by the inhibition of complex I. Taken together with the results in Fig. 2, the decrease of mitochondrial DNA by MPP⁺ may be independent of the inhibition of complex I.

The toxic effects of MPP⁺ have been analyzed in the context of the intracellular ATP depletion and the production of oxygen radicals caused by the inhibition of complex I (3). The cytotoxicity of MPP⁺ analogs is not necessarily correlated to their inhibitory activities on complex I (19). Thus, it is uncertain whether the inhibition of complex I can explain all of the MPP⁺-induced cytotoxicity. The reduction of the copy number of mitochondrial DNA should result in the decrease of the transcripts. The decrease of transcripts in turn should lead to the decrease in the translation products. For example, an antiviral drug zidovudine is reported to inhibit the replication of mitochondrial DNA and cause mitochondrial myopathy resulting from the depletion of mitochondrial DNA (20–22). The decrease of mitochondrial DNA could be another factor related to the deterioration of the mitochondrial respiratory function in Parkinson's disease. It may be of interest to investigate the relationship between the mitochondrial DNA-decreasing effect and the progression of Parkinson's disease-like symptoms by using MPP⁺ analogs.

MPP⁺ decreases the content of mitochondrial DNA in other cell lines, including a neuron-derived cell line,³ indicating that the effect on mitochondrial DNA observed here is a general phenomenon. Because rotenone rather increased the content of mitochondrial DNA, the decrease in mitochondrial DNA by MPP⁺ is not due to the O₂⁻-induced oxidative damage caused by the inhibition of complex I. MPP⁺ is likely to have two independent effects on mitochondria, the inhibition of complex I and the decrease of mitochondrial DNA content. It is intriguing to determine whether the structural part on MPP⁺ responsible for each effect is separated. If there is an MPP⁺ analog which decreases the content of mitochondrial DNA but does not inhibit complex I, the analog may be extremely useful for studying the mechanism for the MPP⁺-induced decrease of mitochondrial DNA content.

Although the mechanism by which MPP⁺ decreases mitochondrial DNA is not clear at the present time, the decrease of the nascent H strand suggests that the replication is inhibited.

³ K. Miyako, Y. Kai, T. Irie, K. Takeshige, and D. Kang, unpublished data.
The inhibition of the replication by zidovudine is due to the delay of the elongation of nascent strand. The delay of the elongation of nascent H strand may accumulate the replication intermediates, which would decrease the nascent H strands per cell, but rather increase the nascent H strands per mitochondrial DNA. Hence, it is likely that the initiation of the replication by MPP is the case. The elucidation of the mechanism should provide a new insight into the regulation of the intracellular content of mitochondrial DNA.

Acknowledgments—We extend special thanks to Drs. Sumimoto and Nakabeppu (Kyushu University) and Prof. Sekiguchi (Fukuoka Dental College) for critical discussion and useful comments. We sincerely thank Prof. Narayanan (New York Medical College) and Prof. Hamasaki (Kyushu University) for the critical reading of the manuscript.

REFERENCES
1. Kopin, I. J., and Markey, S. P. (1988) Annu. Rev. Neurosci. 11, 81–96
2. Burns, R. S., Chiueh, C. C., Markey, S. P., Ebert, M. H., Jacobowitz, D. M., and Kopin, I. J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4546–4550
3. Singer, T. P., and Ramsay, R. R. (1990) FEBS Lett. 274, 1–8
4. Ramsay, R. R., Krueger, M. J., Youngster, S. K., and Singer, T. P. (1991) Biochem. J. 273, 481–484
5. Ramsay, R. R., Kowal, A. T., Johnson, M. K., Salach, J. I., and Singer, T. P. (1987) Arch. Biochem. Biophys. 259, 645–649
6. Ramsay, R. R., Krueger, M. J., Youngster, S. K., Gluck, M. R., Casida, J. E., and Singer, T. P. (1991) J. Neurochem. 56, 1184–1190
7. Takayanagi, R., Takeshige, K., and Minakami, S. (1990) Biochem. J. 192, 853–860
8. Takeshige, K., and Minakami, S. (1979) Biochem. J. 180, 129–135
9. Kang, D., Nahrungashi, H., Sata, T., and Takeshige, K. (1983) J. Biochem. (Tokyo) 94, 1301–1306
10. Hasegawa, E., Takeshige, K., Oishi, T., Murali, Y., and Minakami, S. (1990) Biochem. Biophys. Res. Commun. 170, 1049–1055
11. Przedborski, S., Kostic, V., Jackson-Lewis, V., Naini, A. B., Simonetti, S., Fahn, S., Carlson, E., Epstein, C. J., and Cadet, J. L. (1992) J. Neurosci. 12, 1658–1667
12. Przedborski, S., Jackson-Lewis, V., Yokoyama, R., Shibata, T., Dawson, V. L., and Dawson, T. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4565–4571
13. Kang, D., Nishida, J., Iyama, A., Nakabeppu, Y., Furuta, M., Fujiwara, T., Sekiguchi, M., and Takeshige, K. (1995) J. Biol. Chem. 270, 14659–14665
14. Driggers, W. J., LeDoux, S. P., and Wilson, G. L. (1995) J. Biol. Chem. 268, 22042–22045
15. Clayton, D. A. (1991) Annu. Rev. Cell Biol. 7, 453–478
16. Kadenbach, B., Müncher, C., Frank, V., Muller-Hecker, J., and Napiwotzki, J. (1995) Mutat. Res. 338, 161–172
17. Mueller, P. R. (1992) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Unit 15.5, pp. 1–26, John Wiley & Sons, New York
18. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H., Coulson, A. R., Drouin, J., Ledergerber, I. C., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J., and Young, I. G. (1981) Nature 290, 457–465
19. Kang, D., Miyako, K., Kuribayashi, F., Hasegawa, E., Matsushita, A., Nagano, T., and Takeshige, K. (1997) Arch. Biochem. Biophys. 37, 75–80
20. Arnaudo, E., Dalakas, M., Shanske, S., Moraes, C. T., DiMauro, S., and Schon, E. A. (1991) Lancet 337, 508–510
21. Dalakas, M. C., Atsumi, T., Pezzeshkpoor, G. H., Laskas, J. C., Cohen, B. C., and Griffin, J. L. (1990) N. Engl. J. Med. 322, 1098–1105
22. Moraes, C. T., Shanske, S., Tischler, H. J., Aprile, J., Andreotta, F., Bonilla, E., Shonn, E. A., and DiMauro, S. (1991) Am. J. Hum. Genet. 48, 492–501
23. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560
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J. Biol. Chem. 1997, 272:9605-9608.
doi: 10.1074/jbc.272.15.9605

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