Vol. 143, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1987 Pages 294-299

# INHIBITION OF MITOCHONDRIAL NADH-UBIQUINONE OXIDOREDUCTASE ACTIVITY BY 1-METHYL-4-PHENYLPYRIDINIUM ION

Yoshikuni Mizuno[#], Tomohiko Saitoh[#], and Nobuhito Sone[*]

Department of Neurology[#] and Department of Biochemistry[*],
Jichi Medical School, Tochigi 32904, Japan

Received January 19, 1987

Effect of 1-methyl-4-phenylpyridinium ion ($MPP^+$) on the activity of NADH-ubiquinone oxidoreductase was studied using mitochondria prepared from rat brains. At first, inhibition of oxygen consumption by $MPP^+$ with pyruvate + malate or glutamate + malate as substrates was confirmed polarographically using a Clark-type oxygen electrode. Then, activity of NADH-ubiquinone oxidoreductase in the same samples used in polarography was assayed. Incubation of mitochondria with 0.05 mM of $MPP^+$ together with glutamate, malate and ADP resulted in approximately 50 % inhibition of NADH-ubiquinone oxidoreductase activity. Significance of the results was discussed with respect to the mechanism of neuronal degeneration by $MPP^+$. 

INTRODUCTION. Recently a meperidine analogue, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was found to produce a clinical syndrome quite similar to Parkinson's disease in humans (1). Nigral degeneration and/or loss of striatal dopamine content were confirmed in experimental animals (2-4), and the model has been considered to be the best model available at present of Parkinson's disease (5). An oxidation product of MPTP, 1-methyl-4-phenylpyridinium ion ($MPP^+$), was found to be responsible for the toxicity against dopaminergic neurons (6,7). This oxidation takes place mainly at mitochondrial monoamine oxidase B (8-10). Then, $MPP^+$ is actively taken up into the dopaminergic neurons (7,11). Thus the mechanism of selective toxicity against dopaminergic neurons has been elucidated fairly well, however, the mechanism of neuronal degeneration is still not clear.

We recently reported inhibition of mitochondrial NADH-ubiquinone oxidoreductase [EC 1.6.5.3] activity by $MPP^+$ (12,13). And we thought this might be one of the mechanisms of neuronal degeneration induced by $MPP^+$.

0006-291X/87 $1.50

However, one of the problems was a fact that a rather high concentration of $MPP^+$ (2 to 3 mM) was necessary to induce significant amount of inhibition of this enzyme. Therefore, it was thought that inhibition of NADH-ubiquinone oxidoreductase might not be playing a significant role in inducing neuronal degeneration in vivo. Recently, Ramsay et al. found energy dependent active transport of $MPP^+$ into mitochondria enabling approximately 40-fold concentration (14-16). This observation prompted us to investigate effects of $MPP^+$ on the activity of NADH-ubiquinone oxidoreductase in intact mitochondria preincubated with substrates of the TCA cycle and ADP.

MATERIALS AND METHODS. Two- to 3-month-old male Fisher 344 rats were used. Animals were sacrificed by cervical dislocation, and mitochondrial suspensions were prepared from whole brains according to the method of Ozawa et al. (17). Po pellets were suspended in a medium containing Mannitol (300 mM), KCl (10 mM), potassium phosphate buffer pH 7.5 (10 mM), tris-HCl buffer pH 7.4 (10 mM), EDTA disodium salt (0.2 mM), $MgSO_4$ (4 mM) and 0.2 % BSA. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode in the same medium used to suspend mitochondria. Pyruvate + malate, glutamate + malate and succinate with rotenone (30 nM) were used respectively as substrates at a final concentration of 5 mM. ADP was added at a final concentration of 0.25 mM. After the base line was stabilized, an aliquot of mitochondria (approximately 1 mg protein) was added to a polarography chamber. $MPP^+$ was added simultaneously with mitochondria at a final concentration of 0.05 mM. At the end of polarography, an aliquot of the suspension was used for the assay of NADH-ubiquinone oxidoreductase activity according to the method of Hatefi (18) using a Hitachi-139 spectrophotometer equipped with an isothermal cell holder. Another aliquot was frozen and thawed once, then NADH-ubiquinone oxidoreductase activity was assayed again. Both polarography and enzyme assays were performed at 37° C. Molar extinction coefficient of NADH of 6270 was used for the calculation. Protein was assayed according to the method of Lowry et al. (19). Results were analyzed statistically using the Student's t-test.

RESULTS. Results of polarography are shown in table 1. Oxygen consumption in state 3 and state 4 respirations were significantly inhibited by $MPP^+$ when glutamate + malate or pyruvate + malate were used as substrates. No significant abnormality was noted in state 1 and state 2 respirations. Also, oxidation of succinate was not inhibited by $MPP^+$ at all. These results are consistent with those reported in the literature (20-23).

Results of NADH-ubiquinone oxidoreductase activity in specimens used in polarography with glutamate + malate as substrates are shown in Table 2. Without freeze-thawing, full activity of NADH-ubiquinone oxidoreductase could not be measured. Nevertheless, the activity was significantly inhibited by

Table 1. Effects of $MPP^+$ on the Oxygen Consumption by Mitochondria

| | Glutamate + Malate (n=6) | | Pyruvate + Malate (n=5) | |
|---|---|---|---|---|
| | Control | $MPP^+$(0.05mM) | Control | $MPP^+$ (0.05 mM) |
| State 3 | 75.9 ± 6.8 | 22.8 ± 6.3# | 70.6 ± 12.2 | 20.9 ± 3.0# |
| State 4 | 26.6 ± 3.1 | 17.8 ± 4.3* | 28.8 ± 5.3 | 16.8 ± 3.3* |
| RQ | 2.91 ± 0.61 | 1.30 ± 0.21# | 2.49 ± 0.27 | 1.26 ± 0.14# |
| ADP/O | 2.26 ± 0.07 | 2.01 ± 0.23+ | 2.34 ± 0.10 | 2.09 ± 0.07* |

Expressed as nanomoles of oxygen utilized/min mg protein, mean + SE, RQ = Respiratory quotient, ADP/O = ADP/O ratio, #: $P < 0.001$, *: $P < 0.01$, +: $P < 0.05$.

$MPP^+$ (approximately 50 % inhibition) with the initial concentration of $MPP^+$ of 0.05 mM. The activity was still inhibited in freeze-thawed specimens, but to a lesser degree. Similar assays were attempted with specimens used in polarography with pyruvate + malate as substrates, however, rotenone-resistant oxidation of NADH was significantly elevated with pyruvate, and accurate assays of NADH-ubiquinone oxidoreductase activity were difficult. Ratio of oxygen consumption to the activity of NADH-ubiquinone oxidoreductase is shown in Table 3. Ratio of state 3 respiration was significantly smaller with $MPP^+$ compared with that of the control, however, no significant difference was noted with respect to state 4 respiration.

DISCUSSION. We previously reported inhibition of mitochondrial NADH-ubiquinone oxidoreductase activity by MPTP and $MPP^+$, and suggested that it might be one of the mechanisms of neuronal degeneration in MPTP-induced

Table 2. Effects of $MPP^+$ on NADH-ubiquinone oxidoreductase activity

| | Control | $MPP^+$(0.05mM) |
|---|---|---|
| Non-frozen | 152 ± 32 | 83 ± 14# |
| Freeze-thawed | 241 ± 77 | 177 ± 58* |

Expressed as nanomoles of NADH oxidized/min mg protein. Mean + SE (n=6). Specimens used in polarography with glutamate + malate as substrates served as enzyme sources. #: $P < 0.001$, *: $P < 0.01$ (Paired t-test).

Table 3. Ratio of oxygen consumption to NADH-ubiquinone oxidoreductase activity

| | Control | $MPP^+$(0.05mM) |
|---|---|---|
| State 3 | 1.03 ± 0.18 | 0.56 ± 0.16# |
| State 4 | 0.36 ± 0.10 | 0.43 ± 0.11 |

Expressed as ratio of nanoatoms of oxygen utilized/min mg protein and nanomoles of NADH oxidized/min mg protein measured with non-frozen mitochondria. Mean ± SE (n=6), #: $P < 0.001$.

parkinsonism (12,13). However, the problem was requirement for rather high concentration (mM order) of $MPP^+$ to induce significant amount of inhibition of the enzyme. (In that study, the enzyme activity was assayed using freeze-thawed mitochondria.) The concentration appeared difficult to be reached in vivo. Recently, Ramsay et al. (14-16) discovered an energy dependent transport system for $MPP^+$ in liver mitochondria enabling approximately 40-fold concentration within mitochondria. In the present study, we showed that $MPP^+$ at a relatively low concentration (0.05 mM) could significantly inhibit NADH-ubiquinone oxidoreductase activity by incubating mitochondria with glutamate, malate and ADP. With this concentration of $MPP^+$, inhibition of NADH-ubiquinone oxidoreductase activity could not be seen when assayed with freeze-thawed mitochondria without preincubation (13). In addition, state 1 and 2 respirations were not inhibited. Although we do not have data on intra-mitochondrial concentration of $MPP^+$, our findings appear to be consistent with the observation of Ramsay et al. (14-16) in that mitochondria can concentrate $MPP^+$ by an energy dependent transport system. The concentration of 0.05 mM of $MPP^+$ appears to be what can be obtained in vivo in the substantia nigra of animals treated with MPTP (6,7). Inhibition of NADH-ubiquinone oxidoreductase by $MPP^+$ with resultant decrease in ATP synthesis may be one of the important mechanisms of $MPP^+$- induced neuronal degeneration. Ramsay et al. also reported inhibition of NADH oxidation by inner membranes of mitochondria (22). Apparently, they did not use ubiquinone as an electron acceptor, however, the inhibition of NADH oxidation may well have been due to inhibition of NADH-ubiquinone oxidoreductase. In addition,

Di Monte et al. reported rapid ATP depletion by $MPP^+$ in isolated hepatocytes (24).

When the ratio of oxygen consumption to the activity of NADH-ubiquinone oxidoreductase was analyzed in the absence and presence of $MPP^+$, no significant difference was noted with respect to state 4 respiration. This may be interpreted as that the inhibition of oxygen consumption approximately corresponds to the amount of the enzyme inhibited in state 4 respiration. However, the ratio was significantly lower with respect to state 3 respiration in the presence of $MPP^+$. In another word, inhibition of oxygen consumption is larger than what will be expected from the amount of the enzyme inhibited. Another mechanisms may be operating in addition to the inhibition of NADH-ubiquinone oxidoreductase. Transport of substrates (pyruvate, malate and glutamate) may be inhibited by $MPP^+$. However, it does not seem very likely, because the concentration of the substrates (5mM) used is far higher than that of $MPP^+$ (0.05 mM). Transport of ADP may be inhibited by $MPP^+$. However, this seems unlikely too, because oxygen consumption in state 4 respiration was also inhibited by $MPP^+$. If inhibition of transport of ADP was responsible, respiration after all of ADP was synthesized to ATP, i.e., the state 4 respiration, would have become normalized, which was not the case. It seems to be important to see if there are other enzymes which are inhibited by $MPP^+$ in mitochondria to elucidate the mechanism of $MPP^+$-induced neuronal degeneration.

ACKNOWLEDGEMENTS. This study was supported by the research grant for the investigation of intractable disorders from the Department of Health and Welfare of Japan. Technical assistance from Miss Michiyo Inagawa has been very much appreciated.

REFERENCES

1. Langston, J.W., Ballard, P., Tetrud, J.W. and Irwin, I. (1983) Science 219, 979-980.
2. Burns, R.S., Chiuhe, C.C., Markey, S., Ebert, M.H., Jacobowitz, D. and Kopin, I.J. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 4546-4550.
3. Hallman, H., Olson, L. and Jonson, G. (1984) Eur. J. Pharmacol. 97, 133-136.

4. Heikkila, R.E., Hess, A. and Duvoisin, R.C. (1984) Science 224: 1451-1453.
5. Forno, L.S., Langston, J.W., DeLanney, L.E., Irwin, I., Ricaurte, G.A. (1986) Ann. Neurol. 20, 449-455.
6. Langston, J.W., Irwin, I., Langston, E.B. and Forno, L.S. (1984) Neurosci. Lett. 48, 87-92.
7. Irwin, I. and Langston, J.W. (1985) Life Sci. 36, 207-212.
8. Chiba, K., Trevor, A. and Castagnoli, N., Jr. (1984) Biochem. Biophys. Res. Commun. 120, 574-578.
9. Salah, J.I., Singer, T.P., Castagnoli, N., Jr. and Trevor, A. (1984) Biochem. Biophys. Res. Commun. 125, 831-835.
10. Kinemuchi, H., Arai, Y. and Toyoshima, Y. (1985) Neurosci. Lett. 58, 195-200.
11. Javitch, J.A., D'Amato, R.J., Strittmatter, S.M. and Snyder, S.H. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 2173-2177.
12. Mizuno, Y., Sone, N. and Saitoh, T. (1986) Proc. Japan Acad. 62, Ser B, 261-263.
13. Mizuno, Y., Sone, N. and Saitoh, T. (1987) J. Neurochem. (in press).
14. Ramsay, R.R., Salach, J.I. and Singer, T.P. (1986) Biochem. Biophys. Res. Commun. 134, 743-748.
15. Ramsay, R.R. and Singer, T.P. (1986) J. Biol. Chem. 261, 7585-7587.
16. Ramsay, R.R., Dadgar, J., Trevor, A. and Singer, T.P. (1986) Life Sci. 39, 581-588.
17. Ozawa, K., Seta, K., Takeda, H., Ando, K., Handa, H. and Araki, C. (1966) J. Biochem. 59, 501-510.
18. Hatefi, Y. (1978) Methods in Enzymology, vol. 53 (Fleischer, S. and Packer, L., eds). pp. 11-14, Academic Press, New York.
19. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 258, 2285-2292.
20. Nicklas, W.J., Vyas, I. and Heikkila, R.E. (1985) Life Sci. 36, 2503-2508.
21. Heikkila, R.E., Nicklas, W.J., Vyas, I. and Duvoisin, R.C. (1985) Neurosci. Lett. 62, 389-394.
22. Ramsay, R.R., Salach, J.I., Dadgar, J. and Singer, T.P. (1986) Biochem. Biophys. Res. Commun. 135, 269-275.
23. Vyas, I., Heikkila, R.E. and Nicklas, W.J. (1986) J. Neurochem. 46, 1501-1507.
24. Di Monte, D., Jewell, S.A., Ekström, G., Sandy, M.S. and Smith, M.T. (1986) Biochem. Biophys. Res. Commun. 137, 310-315.