Journal of the Korean Society of
Tobacco Science Vol. 20, No. 1, 99~107 (1998)
Printed in Republic of Korea.

Effect of Cigarette Smoke Exposure on MPTP Metabolism in the Liver of Mice

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(Received May 10, 1998)

ABSTRACT : Numerous studies have demonstrated a negative association between cigarette smoking and Parkinson's disease. The present study was undertaken to investigate whether chronic exposure of mice to cigarette smoke affected the metabolism of 1-methyl-1,2,3,6-tetrahydro-pyridine (MPTP) by cytochrome P-450 (P-450) or flavin-containing monooxygenase (FMO) in the hepatic microsomes of C57BL6/J mice. Adult male C57BL6/J mice were exposed to mainstream smoke generated from 15 cigarettes for 10 min a day and 5 day per week for 6 weeks. MPTP (10 mg/kg body weight) was administered to mice by subcutaneous injection for 6 consecutive days. Microsomal P-450 content was increased by MPTP, smoke exposure, or both, but NADPH cytochrome P-450 reductase activity was rather decreased by the same treatments. The activities of benzo(a)pyrene hydroxylase, 7-ethoxycoumarin *O*-deethylase and ethoxyresorufin *O*-deethylase were significantly increased by the exposure of cigarette smoke, but were not or little affected by MPTP treatment. Benzphetamine *N*-demethylase activity was not affected either by MPTP treatment or by cigarette smoke exposure, but it was significantly increased by the combined MPTP treatment with cigarette smoke exposure, showing their synergic effect for the induction of the enzyme activity. Interestingly, *in vitro* studies of hepatic FMO and P-450 system both *O*-oxygenation and *N*-demethylation of MPTP were increased in the smoke-exposed or in the MPTP-treated mice. These results suggest that the enhancement in the *N*-demethylation as well as *O*-deethylation of P-450 system and in the *N*-oxygenation of FMO activity by cigarette smoke exposure in mouse liver may contribute to attenuating the neurotoxic effects of MPTP on the nigrostriatal dopaminergic neurons.

Key words : **Cigarette smoke exposure, FMO, P-450, MPTP, P-450-dependent monooxygenases**

Most epidemiologic studies have reported that smoker appears to have a lower incidence of Parkinson's disease than nonsmoker (Grandinetti, *et al.*, 1994; Baron, 1995). Several experimental studies have shown that chronic exposure of cigarette smoke has a protective effect against MPTP-induced parkinsonism in an animal model (Carr and Rowell, 1990; Lim *et al.*, 1996; Moon *et al.*, 1997). However, the precise mechanism underlying the protective action of cigarette smoke in the Parkinson's disease process and in the MPTP-induced parkinsonism is not clearly elucidated yet.

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MPTP, a contaminant produced in the synthesis of a meperidine-like narcotic 1-methyl-4-phenyl-4-propionoxypiperidine, causes parkinsonism in humans (Ballard *et al.*, 1985), in fishs (Pollard *et al.*, 1992) and in animals (Gerlach *et al.*, 1991). This agent itself is not neurotoxic, but is metabolized by brain monoamine oxidase-B (MAO-B) to $MPDP^+$, and then to MPP^+ which is potent neurotoxin (Kopin *et al.*, 1988). The metabolic conversion of MPTP to MPP^+ in brain is essential for the expression of neurotoxic action of MPTP in dopaminergic neurons of the substantia nigra. Although brain MAO-B is a key enzyme to convert MPTP to MPP^+, several metabolic pathways have been also reported to exist in the liver. At least, two microsomal enzyme systems are involved in the oxidation of MPTP as an *in vivo* detoxification pathways in the liver: flavine monooxygenase (FMO) (Chiba *et al.*, 1988) and cytochrome P-450 1A1, 2B1, and 3D6 (Ottoboni *et al.*, 1990). Namely, MPTP is also metabolized by liver microsomal FMO to MPTP *N*-oxide and by cytochrome P-450 system to Nor-MPTP.

Cigarette smoke is a complex mixture containing about four thousands of compounds. Cigarette smoking has been associated with stimulation or induction of various hepatic xenobiotic metabolizing enzymes such as cytochrome P-450 related monooxygenase (Pelkonen *et al.*, 1986). Chronic exposure of cigarette smoke to mice may change the rate or pattern in MPTP metabolism by the activation of various xenobiotic metabolizing enzymes in the liver.

We reported previously that cigarette smoke exposure to mice effectively attenuated the decline in the level of striatal dopamine caused by MPTP treatment and that the attenuation resulted from the inhibition of the MAO-B activity, a key enzyme to convert MPTP to neurotoxin MPP^+ in mouse brain (Lim *et al.*, 1996; Sohn *et al.*, 1996; Moon *et al.*, 1997). Although it has known that MPTP is also metabolized by liver microsomal FMO and P-450, the roles of cigarette smoke exposure in the metabolism of MPTP by these two enzyme systems have not been investigated. Therefore, in this study we investigated whether or how cigarette smoke would affect the detoxification rate of MPTP by P-450 or FMO in mouse liver.

MATERIALS AND METHODS

Animals and their treatment. Male C57BL6/J mice weighing 20-22 g were used in this study. The mice were divided into four groups; control group, MPTP-treated group (MPTP), cigarette smoke exposed group (Smoking), and combined MPTP-treated with cigarette smoke exposed group (S-MPTP). Eight mice in each group were housed in a temperature (20±2℃) and light-controlled facility room (12/12 hrs, light/dark cycle) with free access to food and water. Smoking and S-MPTP groups were exposed to mainstream of cigarette smoke generated from 15 filter cigarettes (tar and nicotine content, 11 and 1.1 mg/cigarette, respectively) for 10 min a day, 5 days per week, for 6 weeks using an exposure chamber (D 37 cm x H 13 cm). Smoke exposure was continued until sacrificed. Both MPTP and S-MPTP groups were injected subcutaneously with MPTP (10 mg/kg body weight) once a day for 6 consecutive days from the 4th week to induce neurotoxicity (Carr and Rowell, 1990). Ten days after the final treatment, mice were sacrificed by decapitation. Livers were quickly removed and weighed. The livers were frozen immediately in liquid nitrogen and stored in -70 ℃ until use.

Preparation of liver microsomes. Liver microsomes were prepared from the livers with 30 mM Hepes buffer (pH 7.4) containing 150 mM KCl by differential centrifugation after removing as many contaminants such as blood and other subcellular fractions as possible (Lim *et al.*, 1997). Protein concentrations were determined optically according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Biochemical assays. The activity of FMO in liver microsomes was determined optically by measuring the decrease in absorbance at 340 nm due to oxidation of NADPH under aerobic conditions and in the presence of 3 mM n-octylamine as an inhibitor of cytochrome P-450 as described

by Wu *et al.* (1992). 15 mM MPTP was used as a substrate. The CO-difference spectral method (Omura and Sato, 1964) was used for determining the concentrations of cytochrome P-450 using a mmolar extinction coefficient of 91 $cm^{-1}mM^{-1}$. The activity of NADPH cytochrome *c* (P-450) reductase was measured by monitoring the reduction rate of exogenous cytochrome *c* at 550 nm (Williams and Kamin, 1962). Benzo(a)pyrene hydroxylase activity was measured by the fluorometric assay using 3-OH benzo(a)pyrene as a standard according to the method of Nebert and Gelboin (1968). Determination of ethoxyresorufin *O*-deethylase activity (EROD) was performed by the method of Burke *et al.* (1978). Ethoxycoumarin O-deethylase activity was determined as previously described (Moon *et al.*, 1998) which was modified from the method of Greenlee and poland (1978). Enzyme activities of benzphetamine *N*-demethylase (BPDM) and MPTP *N*-demethylase (MPDM) were determined by using benzphetamine and MPTP as a substrate, respectively, and then by developing the color of formaldehyde formed with Nash reagent according to the method of Tomas *et al.* (1976).

Determination of Nor-MPTP and MPTP N-oxide. The incubation mixture for MPTP metabolism contained 100 mM potassium phosphate buffer (pH 7.4), 0.5 mM $NADP^+$, 2.0 mM glucose-6-phosphate, 1 I.U. of glucose-6-phosphate dehydrogenase, 4 mM $MgCl_2$, 0.1 mM EDTA, 2 mg protein of microsomes and 10 mM MPTP in a total volume of 1 ml. N-octylamine (4.5 mM) was added to the incubation mixture in case of the determination of N-oxygenation activity. The reaction was initiated by the addition of MPTP at 37 ℃ for 10 min in a shaking water bath. The reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid.

Contents of Nor-MPTP and MPTP N-oxide were determined by HPLC analysis as reported by Chiba *et al.* (1988). After the termination of the reaction, quinoline was added to the reaction mixture as an internal standard. The mixture was centrifuged to remove protein at 1,000 x g for 5 min and the supernatants (20 μl) were injected onto the column (4.6 mm x 25 cm) packed with 10 um Partisil 10 SCX (Whatman Chemical Separation Inc., Clifton, NJ, USA). The mobile phase consisted of a mixture of 95% 0.1 M acetic acid and 0.074 M triethylamine HCl, adjusted to pH 2.3 with formic acid, and 5% acetonitrile. Each analyte was monitored at the wavelength of 244 nm. Calibration curves were generated by processing authentic standard substances through the entire procedure. The contents of Nor-MPTP and MPTP N-oxide were calculated by comparison with standard curves using the peak-height ratio method.

Statistics. Data were expressed as the mean ± SD. Analysis of variance (SPSS) followed by Student *t* test was used for determining the statistical significance between the two different groups. A *p* value less than 0.05 was considered to be statistically significant.

RESULTS

Effects of cigarette smoke exposure on *in vivo* MPTP metabolism in the liver. Table 1 shows the changes in the levels of liver microsomal cytochrome P-450 and cytochrome P-450 reductase by MPTP treatment, cigarette smoke exposure, or the combined MPTP-treatment with cigarette smoke exposure to mice. Microsomal P-450 content was increased by MPTP, cigarette smoke exposure, or both, suggesting that both MPTP and cigarette smoke could induce some P-450 isozymes in mouse liver. On the contrary, NADPH cytochrome P-450 reductase activity was rather decreased by the same treatments. But these changes in the each group were not shown with statistical differences compared with the control group.

Table 2 shows the changes in the levels of liver microsomal P-450-dependent monooxygenases by MPTP treatment, cigarette smoke exposure, or the combined MPTP-treatment with cigarette smoke exposure to mice. Benzo(a)pyrene hydroxylase activity was increased by the exposure of cigarette smoke, but decreased by MPTP. Surprisingly, cigarette smoke attenuated the decrease of the

Table 1. Effects of cigarette smoke exposure on the levels of mouse hepatic microsomal cytochrome P-450 and P-450 reductase in MPTP-treated mice

Enzymes	Control	MPTP	Smoking	S-MPTP
P-450 (a)	0.29 ± 0.04	0.33 ± 0.07	0.32 ± 0.08	0.38 ± 0.06
P-450 reductase (b)	26.6 ± 3.6	20.9 ± 1.7	20.3 ± 2.2	20.2 ± 2.1

Eight animals were used in each group. Values are expressed as mean ± SD. S-MPTP: combined MPTP treatment with cigarette smoke exposure. (a) nmole/mg protein (b) nmole/mg protein/min

Table 2. Changes in the levels of liver microsomal cytochrome P-450 monooxygenases by cigarette smoke exposure or MPTP treatment

Enzymes	Control	MPTP	Smoking	S-MPTP
BPDM (a)	404 ± 14	409 ± 24	383 ± 20	663 ± 66**
B(a)P hydroxylase (a)	126 ± 42	112 ± 48	145 ± 29*	123 ± 19
EROD (a)	32 ± 4	33 ± 7	45 ± 4*	39 ± 4
ECOD (b)	309 ± 40	334 ± 35	389 ± 55*	326 ± 30

Eight animals were used in each group. Values are expressed as mean ± SD. S-MPTP: combined MPTP treatment with cigarette smoke exposure. BPDM: benzphetamine *N*-demethylase, EROD: ethoxyresorufin O-deethylase, ECOD: 7-ethoxycoumarin *O*-deethylase. (a) pmoles/mg protein/min, (b) fluorescence arbiturary units

* : Significantly different from the control group ($p<0.05$)

** : Significantly different from the control group ($p<0.01$)

enzyme activity by MPTP. 7-Ethoxycoumarin *O*-deethylase activity was significantly increased by cigarette smoke exposure or by the treatment of MPTP in the mouse. Ethoxyresorufin *O*-deethylase activity was also increased by the exposure of cigarette smoke, but it was not affected by MPTP treatment. Interestingly, benzphetamine *N*-demethylase activity was not affected either by MPTP treatment or by cigarette smoke exposure, but it was significantly increased by the combined MPTP treatment with cigarette smoke exposure, showing their synergic effect for the induction of the enzyme activity or suggesting the induction of a certain P-450 isozyme. These results indicate that P-450 isozymes including P-450 1A were induced by cigarette smoke exposure in mouse liver.

The FMO activity in the liver microsomes is presented in Figure 1. The activities in control, MPTP, Smoking, and S-MPTP groups were 11.2 ± 0.8, 15.0±2.0, 13.0±1.4, and 17.2±1.2 nmoles/min/mg protein, respectively. A significant increase in the FMO activity was observed in both MPTP group ($p<0.05$) and S-MPTP group ($p<0.01$) compared with the control group. Interestingly, although small, increase in the enzyme activity was also shown in S-MPTP group compared with the MPTP group, suggesting the stimulation of MPTP detoxification by cigarette smoke.

Figure 2 shows the change in MPTP *N*-demethylase activity by MPTP treatment or cigarette smoke exposure in the liver. MPTP *N*-demethylase activities in control, MPTP, Smoking, and S-MPTP groups were 1.01±0.04, 1.37±0.15, 1.24±0.14, and 1.58±0.11 nmoles/min/mg protein, respectively. Compared with the control group, metabolic rate of MPTP to MPTP N-oxide was also significantly increased by 36% and 56% in the MPTP ($p<0.01$) and S-MPTP ($p<0.01$) groups, respectively. Interestingly, an

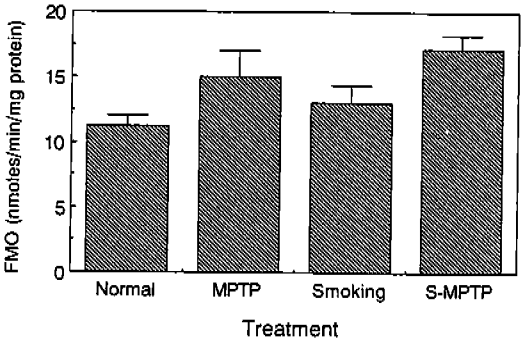

Fig. 1. Effects of cigarette smoke exposure on the liver microsomal FMO activity in the MPTP-treated mice. Data are expressed as mean ± SD of eight mice per group.

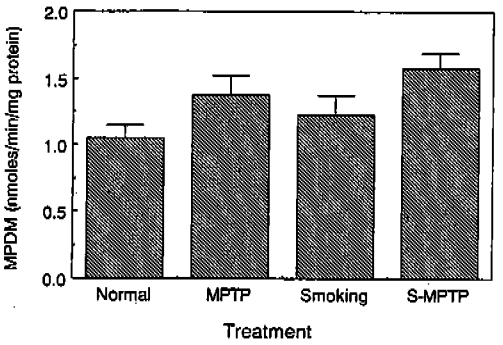

Fig. 2. Effects of cigarette smoke exposure on MPTP N-demethylation in the liver of MPTP-treated mice. Data are expressed as mean ± SD of eight mice per group.

increase in the enzyme activity was also shown in S-MPTP group compared with the MPTP group, suggesting the stimulation of MPTP detoxification by cigarette smoke.

***In vitro* metabolism of MPTP by hepatic microsomes from cigarette smoke-exposed mice.** *In vitro* productions of MPTP N-oxide and Nor-MPTP by liver microsomes prepared from the mice pre-exposed to cigarette smoke are shown in Table 3. It showed that cigarette smoke exposure enhanced the production rate of MPTP N-oxide by 39% and Nor-MPTP by 10% compared with the control, suggesting that cigarette smoke stimulates both *N*-oxygenation and *N*-demethylation of MPTP and that hepatic microsomal FMO system is likely more contributable for detoxification of MPTP than P-450. Analysis of the content of MPTP remained in the reaction mixture after *in vitro* incubation of MPTP with microsomes also showed that cigarette smoke exposure stimulated the MPTP metabolism in mouse liver (Table 3).

DISCUSSION

One of the interesting epidemiological findings in relation to Parkinson's disease is a lower incidence in cigarette smokers than in non-smokers (Grandinetti *et al.*, 1994., Baron, 1995). The relative risk of a smoker in the incidence of Parkinson's disease is approximately 0.5 of nonsmokers (Gresham *et al.*, 1993., Grandinetti *et al.*, 1994). We are pursuing to obtain further new

Table 3. Changes of *in vitro* formations of MPTP N-oxide and Nor-MPTP and MPTP reduction by cigarette smoke exposure in mouse liver microsomes.

Microsomes	MPTP N-oxide (nmol/mg protein/min)	Nor-MPTP (nmol/mg protein/min)	MPTP* (nmol)
Control	4.94 ± 0.23 (100)	3.14 ± 0.37 (100)	25.56 ± 3.86 (100)
Smoke exposed	6.88 ± 0.17 (139)	3.45 ± 0.44 (110)	24.30 ± 3.37 (95)

MPTP N-oxide and Nor-MPTP were formed from MPTP metabolic reaction as described in "Materials and Methods." Values are the mean±SD of eight experiments. The values in parentheses are the percentage of control.

* The contents of MPTP after reaction.

results about a potential mechanism of such production because an understanding of an inverse relationship between Parkinson's disease and cigarette smoking may provide important clues in medical cure for Parkinson's disease. In the present study, we attempted whether cigarette smoke exposure influences hepatic microsomal FMO system and cytochrome P-450, which are responsible for biomodification and detoxification of foreign substances including MPTP.

We first investigated the effects of cigarette smoke exposure on *in vitro* MPTP metabolism in the liver by P-450 or FMO. In general, it has been reported that MPTP is metabolized to Nor-MPTP by hepatic microsomal P-450 1A1, P-450 2B1 and P-450 2D6 (Ottoboni, *et al.*, 1990; Wu *et al.*, 1992; Wu and Ichikawa, 1994 and 1995). In addition, MPTP is also metabolized by the hepatic FMO to MPTP N-oxide (Wu *et al.*, 1992; Wu and Ichikawa, 1994 and 1995). We found that the FMO activity was increased by MPTP treatment and by a combined MPTP treatment with cigarette smoke exposure as well as by cigarette smoke exposure compared with that in the control group. In addition, the N-demethylation of MPTP in liver microsomes was also increased by MPTP administration or cigarette smoke exposure. These observations are in line with previous reports that both hepatic microsomal FMO and P-450 played an important role in the detoxification of MPTP (Wu *et al.*, 1992; Wu and Ichikawa, 1994), and cigarette smoking resulted in increasing the metabolism of xenobiotics, presumably by induction of some P-450 dependent monooxygenases in the liver (Gresham *et al.*, 1993). These findings strongly suggest cigarette smoke exposure to mice can contribute to the detoxification of MPTP by stimulating the hepatic microsomal FMO as well as MPTP *N*-demethylase activity, and these effects become a help to attenuating the neurotoxic effects of MPTP on the nigrostriatal dopaminergic neurons of mice.

We also found that P-450 was increased by MPTP administration, cigarette smoke exposure, or both, in mouse liver microsomes, suggesting both MPTP and cigarette smoke could induce some P-450 isozymes. Our result of P-450 induction by MPTP was not consistent with the results reported previously (Fonne-Pfister *et al.*, 1987; Gresham *et al.*, 1993), which might be due to the different experimental conditions for MPTP treatment each other. Cytochromes P-450 belong to a multigene superfamily (Nelson *et al.*, 1993). It has been known that cigarette smoke differently induces CYP1A, 2B, 2E, and CYP3A (Villard *et al.*, 1998) which play a major role in bioactivation of chemical carcinogens (Gonzalez and Gelboin, 1994). For example, CYP2E1 is induced dramatically by cigarette smoke (Seree *et al.*, 1996; Villard *et al.*, 1998) while CYP2B10 remained unchanged and CYP1A1 is decreased (Villard *et al.*, 1998). It has been reported that MPTP is also metabolized by microsomal P-450 1A1, P-450 2B1, and P-450 2D6 (Ottoboni *et al.*, 1990; Iwahashi *et al.*, 1994). From the reports and our results, we supposed that stimulation of MPTP metabolism by cigarette smoke may result from the induction of CYP2B, CYP2D, not CYP1A.

Our result showed that the activity of NADPH cytochrome P-450 reductase was decreased by MPTP or cigarette smoke as compared with the control group. In fact, there have been a number of reports of differential effects of NADPH cytochrome P-450 reductase on substrate binding and catalysis by some P-450s (Hiroya *et al.*, 1994; Yamazaki *et al.*, 1995; Gruenke *et al.*, 1995). For example, in the case of CYP3A4 the 6β-hydroxylation of testosterone is dependent on P-450 reductase, while the *N*-demethylation of ethylmorphine is not (Yamazaki *et al.*, 1995). There remain further studies whether our results that MPTP or cigarette smoke inhibited the activity of NADPH-cytochrome P-450 reductase affect the metabolism of MPTP to Nor-MPTP by P-450 in mouse liver.

In contrast, benzphetamine *N*-demethylase activity was not affected by MPTP treatment or by cigarette smoke exposure, but it was significantly increased by the combined MPTP treatment with cigarette smoke exposure, showing their synergic effect for the induction of the enzyme activity. This finding suggests that the contribution of cigarette smoke exposure for the detoxification of MPTP is not always associated with the activation

of all enzymes related with cytochrome P-450 system in the liver, but with the stimulation of the *N*-demethylation of MPTP as a cytochrome P-450 dependent monooxygenase.

In the previous study, we confirmed the possibility that long-term exposure of cigarette to C57BL6/J mice attenuates a parkinsonian syndrome induced by the neurotoxin MPTP and such an effect seems to be due to the inhibition of brain MAO-B by unknown compounds in cigarette smoke or its metabolites (Lim *et al.*, 1996). In the present study, we found another possibility at least in part that cigarette smoke exposure had a potential for increasing the MPTP eliminating rate by FMO as well as P-450 in the liver, being capable of supporting an inverse relationship between cigarette smoking and Parkinson's disease. It could be explained from our results that cigarette smoke exposure could contribute to the detoxification of MPTP by liver microsomal FMO or P-450.

In conclusion, cigarette smoke exposure to mice may play an important role in the detoxification of MPTP as a stimulator of FMO as well as P-450 in the hepatic microsomes before MPTP reaches the brain. This function of cigarette smoke may decrease the contents of MPTP delivered to brain for its metabolism by MAO-B, which finally contributes to the protection of dopaminergic nigrostriatal neurons from the MPTP neurotoxicity.

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