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1-Methyl-4-phenyl-pyridinium increases *S*-adenosyl-L-methionine dependent phospholipid methylation

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Abstract

1-Methyl-4-phenyl-pyridinium (MPP^+) and *S*-adenosyl-L-methionine (SAM) cause Parkinson's disease (PD)-like changes. SAM and MPP^+ require their charged *S*-methyl and *N*-methyl groups, so the PD-like symptoms may be related to their ability to modulate the methylation process. The SAM-dependent methylation of phosphatidylethanolamine (PTE) to produce phosphatidylcholine (PTC), via phosphatidylethanolamine-*N*-methyltransferase (PEMT), and the hydrolysis of PTC to form lyso-PTC, a cytotoxic agent, are potential loci for the action of MPP^+. In this study, the effects of MPP^+ on the methylation of PTE to PTC and the production of lyso-PTC were determined. The results showed that SAM increased PTC and lyso-PTC. The rat striatum showed the highest PEMT activity and lyso-PTC formation, which substantiate with the fact that the striatum is the major structure that is affected in PD. MPP^+ significantly enhanced PEMT activity and the formation of lyso-PTC in the rat liver and brain. MPP^+ increased the affinity and the V_{max} of PEMT for SAM. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) effect was lesser and inhibited by deprenyl (MAO-B inhibitor). The nor-methyl analogs of MPP^+ were inactive, but some of the charged analogs of MPP^+ showed comparable effects to those of MPP^+. Lyso-PTC that can be increased by SAM and MPP^+ caused severe impairments of locomotor activities in rats. These results indicate that SAM and MPP^+ have complementary effects on phospholipid methylation. Thus, SAM-induced hypermethylation could be involved in the etiology of PD and an increase of phospholipid methylation could be one of the mechanisms by which MPP^+ causes parkinsonism.

Keywords: Parkinson's disease; Methylation; Phospholipids; MPTP; MPP^+; Phosphatidylcholine; Phosphatidylethanolamine; *S*-adenosyl-L-methionine; Lysophosphatidylcholine

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder. The major symptoms are resting tremors, hypokinesia, rigidity and abnormal posture, caused by the degeneration of dopaminergic neurons in the nigrostriatal pathway and deficiency of dopamine (DA) in the neostriatum (Hornykiewicz, 1966). Norepinephrine (NE) and serotonin (5-HT) levels are also decreased (Bernheimer et al., 1961; Farley and Hornykiewicz, 1976; Scatton et al., 1983; Tohgi et al., 1993), whereas cholinergic activity is increased (Yahr, 1977).

The cause of PD is still not clear although several proposed mechanisms including mitochondrial impairment and oxidative stress have been suggested. An excess of methylation has been proposed to be involved in the etiology of PD because the CNS administration of *S*-adenosyl methionine (SAM), the endogenous methyl donor, causes neurological and biochemical changes that resemble PD (Charlton, 1990; Charlton and Mack, 1994; Charlton and Way, 1978; Crowell et al., 1993). SAM is required for growth and development and to maintain basal metabolic functions, but both the synthesis and the utilization of SAM are increased during aging. Thus, the increase in the limiting factor, SAM, will drive the methylation rate and will result in the metabolism and depletion of L-dopa, DA and other neurotransmitters and contribute to the wear and tear seen during aging. In an individual with the predisposition for PD, such as being endowed with a low prethreshold

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population of DA neurons in the substantia nigra, SAM will precipitate the symptoms of PD. The results from several investigations have also revealed a possible relationship between methylation and parkinsonism. A high N-methylation activity was found in PD patients (Williams et al., 1993). Two endogenous methylated compounds that induce neurotoxicity were found in the parkinsonian brain and were structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is known to cause parkinsonism (Davis et al., 1979; Langston et al., 1984). One toxin is methyl β-carboline, which is known to induce PD-like changes (Collins et al., 1992). The other endogenous neurotoxins are isoquinoline derivatives, which have also been implicated in the cause of PD (Collins, 1994; McNaught et al., 1995). It is noticeable that among isoquinoline derivatives, methylated analogs showed the most potent neurotoxicity (McNaught et al., 1995), suggesting that methylation may be involved in the neurotoxicity of isoquinoline derivatives (Nagatsu, 1997). The neurotoxicity of isoquinoline derivatives is weaker than that of MPTP (Nagatsu, 1997; Nagatsu and Yoshida, 1988; Saitoh et al., 1988; Yoshida et al., 1990).

Methylation is ubiquitous and occurs in diverse biochemical reaction processes. The biological substrates include carbohydrates, proteins, DA, NE and phospholipids. Since the injection of SAM causes PD-like changes, it is conceivable that one or more of those substrates may be responsible for these changes caused by SAM; for example, the excessive methylation of DA may explain the loss of DA, which is the major marker for PD. It has been proposed that the excessive methylation of phospholipids may also play an important role in the pathogenesis of PD because of the structural and biochemical functions controlled by the methylation of phospholipids. Phospholipids are the main components of cell membranes and various biochemical signals such as neurotransmissions and hormonal actions are transmitted through cell membranes. The methylation of phosphatidylethanolamine (PTE) by phosphatidylethanolamine *N*-methyltransferase(s) (PEMTs, 2.1.1.17), using SAM as a methyl donor, increases the production of phosphatidylcholine (PTC). This results in increasing cell membrane fluidity (Hirata and Axelrod, 1978). PTC is hydrolyzed to produce the potent detergent-like cytotoxic compound, lysophosphatidylcholine (lyso-PTC), which can damage plasmalemma and vesicular membranes and may eventually cause cell death if occurring in sufficient quantity. This process also leads to the production of arachidonic acid (AA) and diacylglycerol (DAG) that are powerful cellular mediators and precursors of many important constituents. In addition, PTC and lyso-PTC are precursors of choline and may provide a considerable amount of choline for acetylcholine (ACh) synthesis (Blusztajn and Wurtman, 1983), which may help explain the increase of ACh activity in PD.

MPTP, via 1-methyl-4-phenyl-pyridinium (MPP^+), the oxidized metabolite of MPTP, causes substantia nigra cell death and parkinsonism (Davis et al., 1979; Langston et al., 1984; Russ et al., 1991). Thus, the elucidation of the mechanism of MPTP/MPP^+ toxicity may provide clues to the pathogenesis of substantia nigra cell death in PD. A number of mechanisms have been proposed for the toxicity of MPTP/MPP^+ (Fornai et al., 1996; Kupsch et al., 1996). The impairments of mitochondrial function (Nicklas et al., 1985; Mizuno et al., 1987; Youngster et al., 1989) and oxidative stress (Halliwell, 1992) have been preferentially projected to explain the mechanism of MPTP/MPP^+ toxicity. However, not all studies are in agreement. For example, evidence shows that damage to complex I and/or oxidative stress cannot be the cause of the MPTP/MPP^+-induced effects on dopaminergic neurons in the primate brain (Gerlach et al., 1995). One reason is that despite severe PD-like changes, MPTP treatment was shown to have no effect on any of the enzymes of the respiratory chain and indices of oxidative damage in nigrostriatal region. Apoptosis (Dipasquale et al., 1991; Mochizuki et al., 1994), *N*-methyl-D-aspartate (NMDA) receptor-mediated calcium influxes (Fornai et al., 1996; Mehta and Ticku, 1990), the involvement of cholinergic nervous system (Hadjiconstantinou et al., 1994), hypothermia (Eaker et al., 1987; Freyaldenhoven et al., 1995) and disturbance of intracellular calcium homeostasis (Kass et al., 1988; Kupsch et al., 1996) have also been suggested for MPTP/MPP^+ neurotoxicity. Although a number of hypotheses for the neurotoxicity of MPTP/MPP^+ have been proposed, most of the proposed mechanisms might not be exclusive; rather they may be consequential events.

The interaction of MPP^+ with the SAM-dependent methylation process may constitute one of the mechanisms by which MPP^+ causes PD-like changes, because SAM causes PD-like changes when placed into the rat brain. More interestingly, like SAM, which requires the charged *S*-methyl group, MPP^+ also requires the charged *N*-methyl group for its activity. As a xenobiochemical, MPP^+ therefore may modulate the endogenous SAM-dependent methylation process. In the present study, the effects of MPTP and MPP^+ on phospholipid methylation were investigated to support the hypermethylation hypothesis for PD and help explain the mechanism of MPTP/MPP^+-induced neurotoxicity.

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN) and kept in a colony room for at least 5 days before the beginning of the experiments. The animals had access to food and water ad libitum, under a 12-h light/dark cycle. MPTP·HCl and MPP·I were obtained from RBI (Natick, MA). 4-Phenyl-1,2,3,6-tetrahydropyridine (4-PT), 4-phenyl-pyridine (4-PP), 1-ethyl-4-phenyl-pyridinium (EPP^+), 1-propyl-4-phenyl-pyridinium (PPP^+), 1,4-

dimethyl-pyridinium (DMP^+) and 1-cetyl-pyridinium (CP^+) were purchased from Aldrich Chemical Company (Milwaukee, WI). [^{3}H-methyl]-*S*-adenosylmethionine (SAM) was from New England Nuclear (Boston, MA) and silica gel thin layer chromatography (TLC) plate (LK5D) was from Fisher Scientific (Pittsburgh, PA). All other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Tissue preparation

Male Sprague–Dawley rats weighing 250–350 g were decapitated and the brain and liver tissues were homogenized (5 ml/g tissue) using a Polytron (Model: PT 3100) in 50 mM Tris–HCl buffer, pH 9.5, containing 15 mM Mg^{2+} and 20 mM EDTA. The rat brain synaptosomal fraction was prepared by a modification of the previous method (Fuxe et al., 1967). In brief, the rat brain tissue was homogenized (10 ml/g tissue) in 0.32 M sucrose, followed by centrifugation at $1000 \times g$ for 10 min to remove tissue debris and nuclei. The supernatant was centrifuged again at $20{,}000 \times g$ for 10 min to obtain the mitochondrial fraction. The pellet, crude mitochondrial fraction, was diluted in 0.32 M sucrose to have an approximate 10 volumes of final concentration and applied to a discontinuous density gradient centrifugation in which the lower phase contained 9 ml of 1.2 M sucrose and the top phase contained 3 ml of 0.8 M sucrose. The tissue preparations in 0.32 M sucrose were loaded on the top of 0.8 M sucrose phase and centrifuged at $100{,}000 \times g$, 4°C, for 60 min. The fractions at the interface of 0.8 and 1.2 M sucrose were collected (synaptosomal fraction) and stored at −70°C until assays were performed.

2.3. PEMT assay

The procedures described previously (Hirata et al., 1978) were used with some modifications. PEMT activity was measured in the medium, containing tissue preparation (0.2–1.5 mg protein), 50 mM Tris–HCl buffer, 15 mM Mg^{2+}, 20 mM EDTA and SAM at pH 9.5 or 7.5 in a total volume of 100 μl. MPTP·HCl, MPP·I, or their analogs were added to some tubes before incubation. The reaction was started by the addition of [^{3}H-methyl]SAM and continued for 1 h at 37°C. The reaction was terminated by the addition of 3 ml of chloroform: methanol: HCl (100:50:1, v/v/v). Lipids were extracted for 10 min and washed twice with 2 ml of 0.1 M KCl in 50% methanol. The chloroform phase that contained the phospholipids was dried under nitrogen gas, extracted with 50 μl of chloroform: methanol (2:1, v/v), and applied to TLC plate for the separation of methylated phospholipids.

2.4. Identification of reaction products by TLC

Individual phospholipid products of the reaction were analyzed by applying aliquots of the organic phase to a silica gel TLC plate (LK5D silica gel). The mobile phase was *n*-propanol:propionic acid:chloroform:water (2:2:1:1, v/v/v/v) or chloroform:methanol:water (65:25:4, v/v/v). Standard phospholipids dissolved in chloroform:methanol (2:1) were also chromatographed. The chromatograms were visualized by exposing the plate to iodine vapor. The spots that matched the standards were scraped off the plates and solubilized in scintillation cocktail and the radioactivity was measured by scintillation counting.

2.5. Animal preparation for the measurement of locomotor activities

Sprague–Dawley male rats weighing 250–350 g were anesthetized with chloral hydrate (400 mg/kg, ip) and a 22-gauge stainless steel cannula was stereotaxically implanted into the skull for injection into the lateral ventricle of each rat. The placement of the cannula, with reference to bregma, was 1.4 mm lateral and 0.6 mm caudal, and the tip extended into the cortex over lateral ventricle. The cannula was affixed with dental cement and secured to the skull with two screws. A stainless steel rod was inserted into each cannula to maintain its opening. The rats were allowed to recover for about 2–3 days before the experiments. Injection

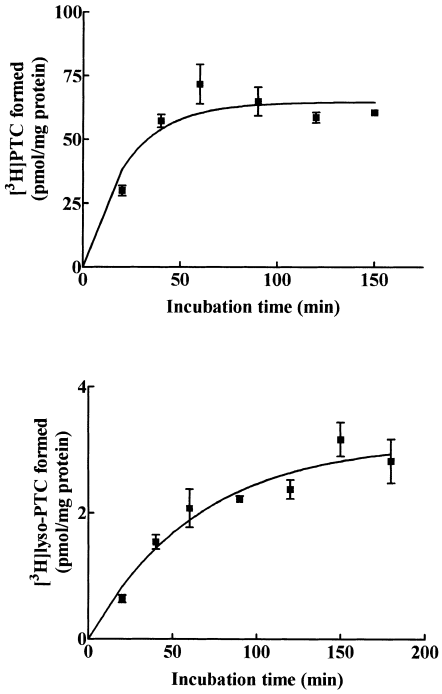

Fig. 1. Time-dependent study of phospholipid methylation and the formation of lyso-PTC in rat liver homogenate. Rat liver tissue homogenate was incubated in 50 mM Tris–HCl buffer, 15 mM Mg^{2+} and 20 mM EDTA, 100 μM SAM containing 0.55 μCi of [^{3}H-methyl]SAM (55.1 Ci/mmol of specific activity), pH 7.5, in a total volume of 200 μl at 37°C for 1 h. After the reaction was terminated by the addition of 3 ml of chloroform:methanol:HCl (100:50:1), the lipids were extracted and analyzed by TLC as described in the Materials and Methods section. Results shown are the means ± S.E.M. ($n = 3$). These results are from three experiments.

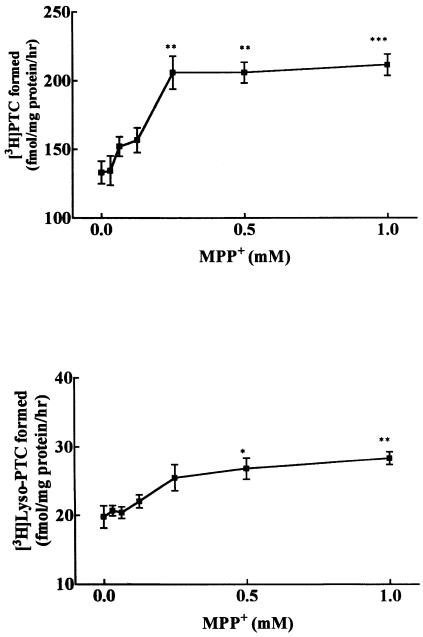

Fig. 2. Effect of MPP^+ on SAM-dependent methylation of PTE to phosphatidylcholine (PTC) and the formation of lyso-PTC in rat brain synaptosomal fractions. Aliquots of tissue were incubated with various concentrations of MPP^+ (0–1 mM) and 100 nM of [^{3}H-methyl]SAM (0.55 μCi, 55.1 Ci/mmol of specific activity) in 50 mM Tris–HCl, containing 15 mM Mg^{2+}, 20 mM EDTA, pH 9.5, in a total volume of 100 μl at 37°C for 1 h. The measurement of the incorporation of [^{3}H]methyl groups from [^{3}H]SAM into phospholipids was described in the Materials and Methods section. The yield of methylated phospholipids was dependent on the amount of SAM used for the experiment. Results shown are the means ± S.E.M. ($n=3$). These results are from three experiments. * Indicates significant increases when compared to the control (* $P<.05$, ** $P<.01$, *** $P<.001$) using Student's t test and one-way ANOVA test.

was made in the right lateral ventricle 5 mm from the surface of the cranium, via a premeasured insertion cannula, attached by polyethylene tubing (PE 20) to a 10-μl Hamilton syringe.

2.6. Measurement of locomotor activities

Lyso-PTC was dissolved in phosphate-buffered saline (PBS), pH 7.4. The stainless steel rod was removed and the rats were injected with 5 μl of PBS, as a control, or different concentrations of lyso-PTC (intracerebroventricularly). The motor activities of the animals were measured at 2 min postinjection for 30 min, using an activity monitor (Digiscan Instruments, Columbus, OH). Total distance (TD) traveled, the number of movements (NM), and movement time (MT) were determined.

2.7. Protein assay

The protein concentration was determined using Pierce BCA protein assay kit (Rockford, IL) with bovine serum albumin as the standard and absorbance was measured at 562 nm.

2.8. Statistical analysis

The mean and standard error of the mean (S.E.M.) were determined for each set of data and two-tailed Student's t test and one-way ANOVA followed by post hoc Bonferroni test were used for statistical analysis to compare control and treated groups. A probability of less than .05 was considered as a significant difference.

3. Results

3.1. The time course study of phospholipid methylation and the formation of lyso-PTC

The first set of studies involved the verification of the reaction process for the methylation of PTE to PTC using the rat liver tissue homogenates. The time-course effects and whether lyso-PTC was correspondingly increased were examined. Time-dependent studies revealed that the formations of PTC and lyso-PTC were initially linear and plateaued after about 60 and 100 min, respectively (Fig. 1), and were consistent with the previous report (Tsvetnitsky et al., 1995). The methylation of PTE to form PTC and lyso-PTC was dependent on the amount of SAM used for the assay.

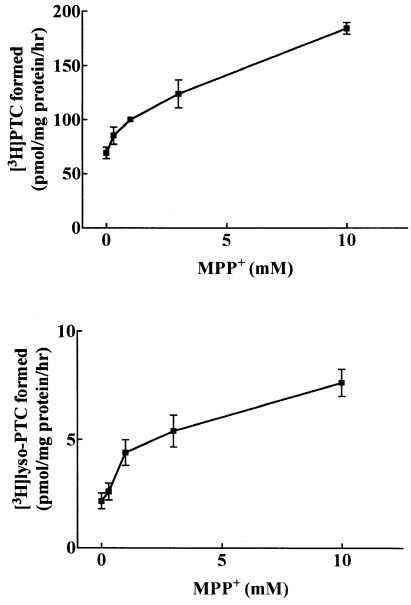

Fig. 3. Effect of MPP^+ on the SAM-dependent methylation of PTE to PTC and the formation of lyso-PTC from PTC in rat liver homogenate. The rat liver tissue homogenate was incubated with 50 μM SAM containing 0.28 μCi [^{3}H-methyl]SAM and various concentrations of MPP^+ in 50 mM Tris–HCl, containing 15 mM Mg^{2+}, 20 mM EDTA in a total volume of 100 μl for 1 h at 37°C. The lipids were extracted and separated by thin layer chromatography as described in the Materials and Methods section. The values shown are the means ± S.E.M. for triplicate samples. These results are from two experiments.

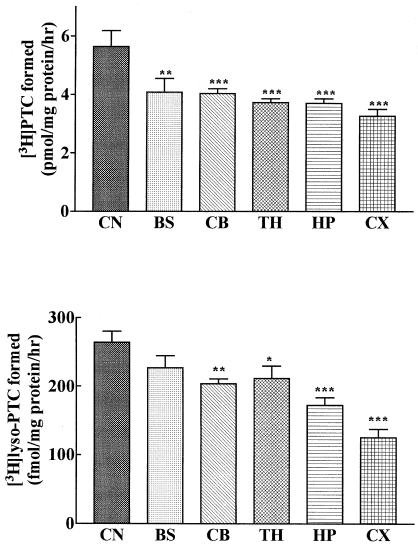

Fig. 4. PEMT activity and the formation of lyso-PTC in different regions of rat brain. Each region of rat brain was dissected and homogenized in 5 volumes of Tris–HCl buffer at pH 9.5. Aliquots of tissue (200–300 μg protein) were incubated with 50 μM SAM containing 0.55 μCi [^{3}H-methyl]SAM for 1 h at 37°C. After the reaction was terminated by the addition of 3 ml of chloroform:methanol:HCl (100:50:1), methylated phospholipids were measured as described in the Materials and Methods section. Values are expressed as means ± S.E.M. (n = 7). * Indicates significant differences when compared to the CN region (* P < .05, ** P < .01, *** P < .001) using Student's t test and one-way ANOVA test. CN: caudate nucleus, BS: brain stem (midbrain, pons and medulla oblongata), CB: cerebellum, TH: thalamus/hypothalamus, HP: hippocampus, CX: cortex.

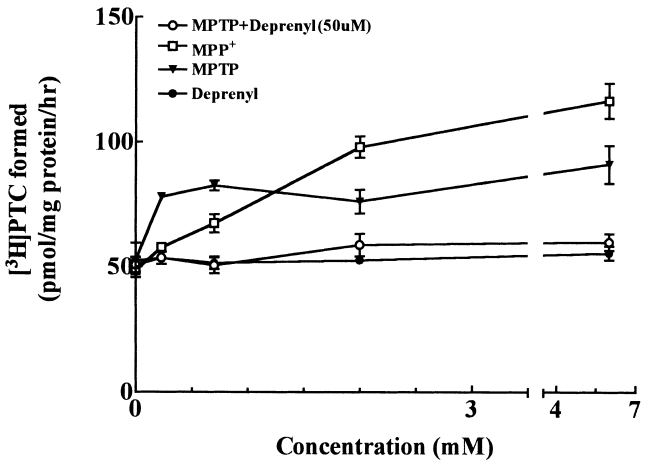

Fig. 5. The effects of MPP$^+$ (□), MPTP (▼), deprenyl (●), or MPTP plus deprenyl (50 μM) (○) on PEMT activity in the rat liver homogenate. The rat liver tissue homogenate and 50 μM SAM containing 0.28 μCi [^{3}H-methyl]SAM were incubated with MPTP, MPP$^+$, deprenyl, or MPTP plus deprenyl for 1 h at 37°C in a total volume of 100 μl. The lipids were extracted and separated as described in the Materials and Methods section. The [^{3}H]methyl phospholipids were measured by scintillation counter. Each point represents the means ± S.E.M. for triplicate samples from three experiments. * P < .05 compared to control using Student's t test and one-way ANOVA test.

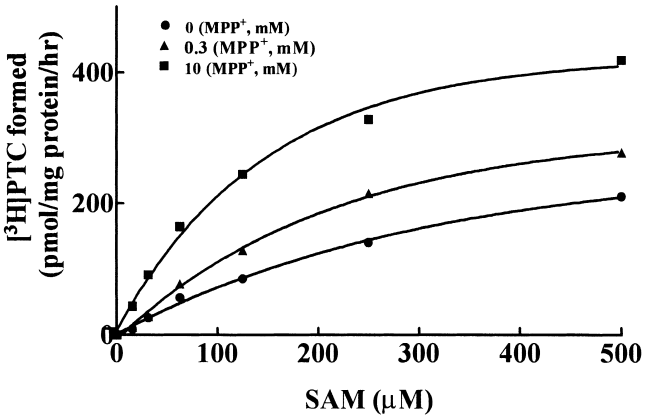

Fig. 6. The effect of MPP$^+$ on the kinetics of PEMT. Aliquots of the rat liver tissue (300 μg protein) were incubated with various concentrations of SAM, 50 nM [^{3}H]SAM at 37°C for 1 h at pH 9.5. Each set of samples was incubated under the different concentrations of MPP$^+$ (0–10 mM). Concentrations of SAM tested were 15.6, 31.2, 62.5, 125, 250, 500 μM. Results shown are the means ± S.E.M. (n = 3) from three experiments.

3.2. Effect of MPP$^+$ on phospholipid methylation in rat brain and liver tissue

The present study showed that MPP$^+$ increased phospholipid methylation in the rat brain synaptosomal fraction and liver tissue homogenate. MPP$^+$, in a concentration-dependent manner, increased the methylation of PTE to form PTC and also increased the formation of lyso-PTC in rat brain (Fig. 2) and liver tissue homogenate (Fig. 3). The yield of methylated phospholipids was dependent on the amount of SAM used for the assay. Concentrations of 0.25, 0.5 and 1.0 mM of MPP$^+$ increased the methylation of PTE to PTC by 54%, 55% and 59%, respectively, and also significantly increased the formation of lyso-PTC by 29%, 36% and 43%, respectively, in rat brain as indicated by the production of PTC. MPP$^+$ increased PEMT activity and also lyso-PTC formation by several folds in rat liver.

3.3. PEMT activity in various regions of rat brain

Fig. 4 shows the methylation of PTE to form PTC and the formation of lyso-PTC from methylated PTC in various

Table 1
The effect of MPP$^+$ on the kinetics of PEMT

MPP$^+$ (mM)	K_m (μM)	V_{max} (PTC, pmol/mg protein per hour)
0	135 ± 38.2	198 ± 29.6
0.3	118 ± 22.6	275 ± 35.9
1	105 ± 16.8	295 ± 26.8
3	98 ± 15.2	310 ± 56.5
10	85 ± 11.4	400 ± 45.9*

Michaelis–Menten kinetics was applied to analyze data. Values are the means ± S.E.M. (n = 3) from three experiments. * P < .05 compared to control using Student's t test and one-way ANOVA test.

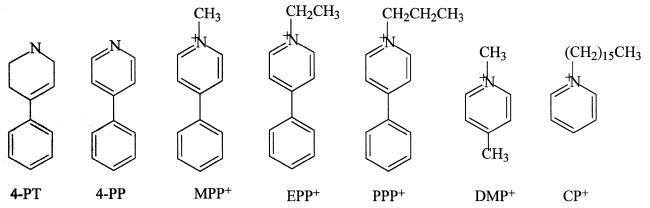

Fig. 7. Structures of MPP^+ and its analogs.

brain regions. These reactions are indices of the activity of PEMT. The results showed that the striatum (caudate nucleus) revealed the highest PEMT activity. As compared to the cortex, the region with the lowest activity, the striatum has about 42% higher PEMT activity. The brain stem region that contains the midbrain, pons and medulla oblongata also showed higher PEMT activity. The midbrain contains substantia nigra. This implies that events that require PEMT may have its greatest effects in the striatum. Consequently, SAM and probably MPP^+ may show the greatest effects in the striatum. Since dopaminergic nerve terminals in the striatum are degenerated in PD, these findings may be consistent with the previously published observations that SAM and MPP^+ cause PD-like effects (Burns et al., 1983; Crowell et al., 1993).

3.4. MPP^+, not MPTP, is the active compound for enhancing PEMT activity

Since MPTP induces parkinsonism and hypermethylation also causes PD-like changes, it was speculated that MPTP might cause parkinsonism through increasing methylation. MPTP, however, induces parkinsonism via its metabolite, MPP^+. Therefore, the experiment was designed to determine whether MPTP needed to be oxidized to MPP^+ for enhancing PEMT activity. Various concentrations of MPTP, MPP^+, deprenyl or MPTP plus deprenyl were incubated with SAM in rat liver homogenate. Deprenyl was used to inhibit the oxidation of MPTP to MPP^+. As shown in Fig. 5, MPP^+, in a concentration-dependent manner, increased the production of PTC from PTE. At lower concentrations, MPTP caused higher increments in PTC than MPP^+, but at higher concentrations of MPTP, the effect subsided or

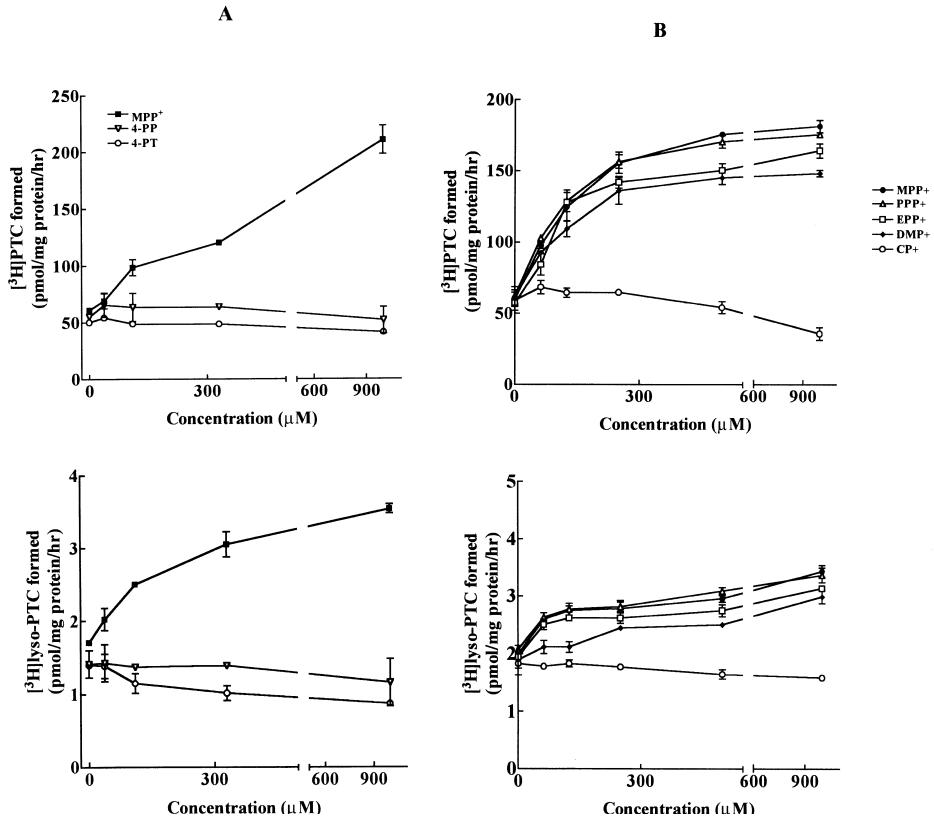

Fig. 8. Comparison of the MPP^+ effect on PEMT activity with its analogs. Aliquots of the rat liver tissue homogenate were incubated with various concentrations of MPP^+ analogs and 50 μM SAM containing 0.28 μCi [^{3}H-methyl]SAM under standard conditions as described in the Materials and Methods section. Each point represents the means ± S.E.M. for triplicate samples from two experiments. (A) Effect of uncharged nor-methyl MPP^+ analogs on PEMT activity. MPP^+: 1-methyl-4-phenyl pyridinium, 4-PP: 4-phenyl-pyridine, 4-PT: 4-phenyl-1,2,3,6-tetrahydropyridine. (B) Effect of charged MPP^+ analogs on PEMT activity. EPP^+: 1-ethyl-4-phenyl-pyridinium, PPP^+: 1-propyl-4-phenyl-pyridinium, DMP^+: 1,4-dimethyl-pyridinium, CP^+: 1-cetyl-pyridinium.

plateaued. Deprenyl, a monoamine oxidase-B inhibitor (MAOB-I), did not show any effect on methylation. The addition of 50 μM of deprenyl with MPTP blocked the MPTP response. The data suggest that MPP^+ is the active compound. The inhibition of the oxidation of MPTP to MPP^+ by deprenyl may be responsible for blocking the effect of MPTP on PEMT activity. This result is consistent with the fact that parkinsonism, seen following MPTP administration, is dependent on the conversion of MPTP to MPP^+ and that deprenyl blocked this oxidation process.

3.5. Effect of MPP^+ on PEMT kinetics

The mechanism for the action of MPP^+ on SAM-dependent phospholipid methylation was investigated. To do so, the effects of MPP^+ on the SAM-dependent synthesis of PTC were tested using rat liver tissue homogenate. Shown in Fig. 6 are the effects of 0.0, 0.3 and 10 mM MPP^+ on PEMT activity. Both the affinity and the maximal level of the reaction were increased in a concentration-dependent manner by MPP^+. The Michaelis–Menten kinetics of PEMT for the SAM-dependent synthesis of PTC was also determined. Table 1 shows the relative changes in both the K_m and V_{max} of PEMT for SAM following doses of 0.0, 0.3, 1, 3 and 10 mM of MPP^+. Using radioactive SAM, it was shown that the control kinetic values (K_m and V_{max}) of PEMT for the methylation of PTE to form PTC were K_m value of 135 μM and V_{max} of 198 pmol/mg protein/hr. The respective concentrations of MPP^+ increased the affinity of PEMT for SAM, as evident by the decrease in the K_m from 135 to 118, 105, 98 and 85 μM, respectively. The V_{max} was increased from 198 to 275, 295, 310 and 400 pmol/mg protein per hour by the above doses of MPP^+. Thus, 10 mM of MPP^+ decreased the K_m by 41% and increased the V_{max} by 102%.

3.6. Effect of MPP^+ analogs on SAM-dependent synthesis of PTC

It was of interest to determine whether the charged *N*-methyl group was involved in the ability of MPP^+ to increase the methylation of PTE to form PTC and the eventual production of lyso-PTC by testing the analogs of MPTP/MPP^+ (Fig. 7). This may be relevant to the fact that SAM also has a charged methyl group and both SAM and MPP^+ induce PD-like changes. The uncharged nor-methyl analogs of MPP^+ and MPTP, 4-PP and 4-PT were compared with MPP^+ for their abilities to increase the SAM-dependent production of PTC and lyso-PTC (Fig. 8A). MPP^+, in a concentration-dependent manner, increased the production of PTC and lyso-PTC. One mM of MPP^+ increased PTC and lyso-PTC by 300% and 200%, respectively. However, the uncharged nor-methyl analogs, 4-PP and 4-PT, had no effect on PEMT activity. The charged analogs of MPP^+ were EPP^+, PPP^+, DMP^+ and CP^+; all have substituents on the nitrogen atom in the pyridinium ring. Except for CP^+, they all showed comparable effects on PEMT activity similar to MPP^+ (Fig. 8B). CP^+, which is charged, but has a long carbon chain on the nitrogen and lacks C_4 substitution, showed the opposite effect, which slightly decreased PEMT activity. The results indicate that the charged pyridinium moiety is required for the activity of the analogs, but it can be offset by a bulky or long-chain substituent or the absence of the C_4 substituent. These results are consistent with previous study of MPP^+ analog-induced neurotoxicity (Rollema et al., 1990).

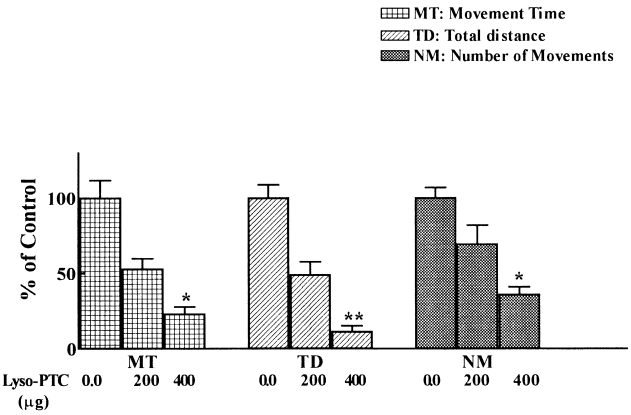

Fig. 9. Effect of lyso-PTC on locomotor activities in rats. After rats were cannulated as described in the Materials and Methods section, lyso-PTC was injected into the lateral ventricle of rat brain. Measurements were made for 30-min post injection time in different groups of rats. Movement times, total distance and number of movements by rats pretreated with 5 μl of PBS, lyso-PTC 200 μg or 400 μg (intracerebroventricularly) were measured. Data are expressed as means ± S.E.M. ($n=5$) from two experiments. Two tailed Student's t test and one-way ANOVA followed by post hoc Bonferroni test were used for statistical analysis to compare control and treated groups. * Indicates significant decreases when compared to the control ($*P<.05$, $**P<.01$).

3.7. Effect of lyso-PTC on locomotor activities in rats

The present study showed that SAM and MPP^+ increased lyso-PTC, a detergent-like cytotoxic compound, via increasing PTC production, therefore part of their PD-like effects may be transduced through the action of lyso-PTC. Because the major symptoms of PD are dysfunctions of movements, the effects of lyso-PTC on locomotor activities were investigated. The injection of 200 and 400 μg of lyso-PTC into the lateral ventricle of the rat brain caused marked (approximately 50 and 75%) reduction of motor functions. In a dose-dependent manner, lyso-PTC caused severe hypokinesia as measured by the decrease on movement time (MT), total distance (TD) and number of movements (NM) (Fig. 9). These impairments of locomotor activities by lyso-PTC may contribute to the SAM-induced and MPP^+-induced PD-like symptoms. Thus, the increase in lyso-PTC may be a common underlying cause for other changes such as neuronal death that are seen following the injection of SAM and MPP^+.

4. Discussion

The results from the present study showing that MPP^+ enhances phospholipid methylation and increases lyso-PTC suggest that the mechanism by which MPTP — via MPP^+ — causes parkisonism is, at least in part, by increasing methylation.

SAM increases the production of PTC and lyso-PTC in a concentration-dependent manner in rat liver homogenate, as well as in brain tissue (Crews et al., 1980). PTC is a major methylated phospholipid with membrane forming and emulsifying properties and lyso-PTC is a detergent-like cytotoxic compound. Lyso-PTC may exhibit its effects on the local tissue where it is produced (Yuan et al., 1995). This is interesting because the rat striatum showed the highest PEMT activity and the highest rate of lyso-PTC formation. If SAM is involved in PD, therefore, excessive methylation could result in the production of high levels of PTC and lyso-PTC in the striatum. Lyso-PTC could damage dopaminergic neuronal terminals and cause retrograde damage of cell bodies in the substantia nigra as has been observed in our unpublished study. Interestingly, the mechanism for MPTP damage to nigrostriatal DA neurons was also proposed to involve retrograde degeneration (Davis et al., 1979).

Our studies show that MPP^+ increases phospholipid methylation. Although MPTP has an initial effect, MPTP did not increase phospholipid methylation in the presence of deprenyl, an MAO-B inhibitor. The initial lower dose-responsiveness for MPP^+, as compared to MPTP, may be related to the rate of transport of MPTP and MPP^+ across lipid bilayers. Micelles might have been formed during homogenizing, embedding PEMT in its interior surfaces. Unlike the charged MPP^+ molecule, at lower concentrations the uncharged MPTP molecule may gain ready access to the inner membranes of micelles, and undergoes limited oxidization to MPP^+. Deprenyl, which inhibits the oxidation of MPTP, blocks this effect of MPTP.

The exact mechanism by which MPP^+ enhances PEMT activity is not clear from the present study. MPP^+ decreased the K_m of PEMT for SAM, thus increasing its affinity for SAM. The increased affinity of PEMT for SAM by MPP^+ may be the result of allosteric effect on the enzyme. MPP^+ also increased the V_{max} of PEMT, indicating that MPP^+ does not compete with SAM for the binding site on PEMT. PEMT may have a regulatory site that has a strong affinity for the more stable MPP^+, whereas the catalytic site favored the more labile (methyl-leaving) SAM. The proposed regulatory site on PEMT is not specific for the S^+-CH_3 or N^+-CH_3 groups, because other analogs with ethyl and propyl substituents also increase the effect of SAM. While PEMT is regulated by G-proteins and protein kinase (Morrill, et al., 1994; Villalba et al., 1987), further investigation will be required to elucidate the mechanism of MPP^+-enhanced PEMT activity.

The study, however, suggests that MPP^+ and its analogs need to be charged and have short substituents on the nitrogen atom in the pyridinium ring to enhance PEMT activity. Two MPTP/MPP^+ analogs (4-PP and 4-PT), which are uncharged and have no substituent on nitrogen, did not increase the methylation process. On the other hand, the long carbon chain substituent on nitrogen in pyridinium, as shown for CP^+, decreased PEMT activity, although it is a charged molecule. Further investigation is required to establish the relationship between molecular structures of analogs and PEMT activity because the lack of a substituent on the C_4 position of CP^+ may also be involved. The results, nonetheless, may help explain the observation that the *N*-methyl group is required for the toxicity of MPTP (Yoshida et al., 1990) and that the demethylated analogs of MPTP were devoid of toxicity under the conditions in which MPTP resulted in significant toxicity (Duvoisin et al., 1986).

PD is a CNS disorder, but the systemic effects of MPP^+ on phospholipid methylation cannot be ignored because there is some evidence that systemic lipids pass the blood–brain barrier (BBB) and affect brain functions. It has been reported that PTC formed in the liver rapidly reaches equilibrium with the plasma (Bjornstad and Bremer, 1966), whose phospholipids are derived mainly from the liver (Fishler et al., 1943). The preceding information suggests the possible transportation of systemic phospholipids to the brain, noting that PTC intake increases ACh in the rat brain (Hirsch and Wurtman, 1978). This shows that PTC from the systemic circulation can be a source for choline in the synthesis of ACh in the brain. Lyso-PTC, a detergent-like cytotoxic compound, is able to penetrate the blood–brain barrier (BBB) (Alberghina et al., 1994). It is also water soluble, and therefore it may accumulate in the CSF, which is adjacent to a large surface of the striatum, hippocampus, and brain stem. The dramatic increment of PTC and lyso-PTC by MPP^+ in the liver tissue, as shown in this study, means that the phospholipids may be transported to and affect the brain. Thus, increased phospholipid methylation in the brain, as well as peripheral systemic tissues may help to explain the increased cholinergic activity in PD. This speculation of MPP^+-enhanced phospholipid methylation-increased cholinergic activity is supported by the previous study (Cavalla et al., 1985) in which treatment with MPTP or MPP^+ increased choline and ACh levels. Although they did not explain the mechanism by which MPTP increased choline and ACh production, it may be associated with an increase of phospholipid methylation. The demethylated MPTP analogs, 4-phenyl pyridine and 4-phenyl 1,2,3,6-tetrahydropyridine, did not affect either the levels of choline or ACh (Cavalla et al., 1985).

There are two ways to synthesize PTC. One is the CDP (cytidine diphosphate)–choline pathway and the other is the PEMT pathway. Although CDP-choline pathway is the major route for the synthesis of PTC, the PEMT pathway is the only known pathway that synthesizes de novo choline molecules. This underscores the important physiological role of the PEMT pathway. Furthermore, the PTC mole-

cules formed by this N-methylation are enriched with polyunsaturated fatty acids including AA (Ridgway and Vance, 1987).

Another significant consequence of increasing methylation of phospholipids is the fact that the elevation of PTC will generate not only lyso-PTC, but also DAG, free fatty acids including AA and other metabolites. These products are closely associated with the transmission of many signals through membranes (Hirata and Axelrod, 1980). Accumulation of free AA itself and/or its further metabolism to eicosanoids may affect membrane permeability and neuronal function (Bazan et al., 1993, 1995). The present study showed that lyso-PTC caused severe impairments in motor functions that may be one of the mechanisms through which MPP^+ and SAM induced PD-like symptoms. Elevated level of lyso-PTC is known to be involved in atherosclerosis and inflammation process; therefore, lyso-PTC, which is increased by excessive methylation, or MPP^+, may cause pathological conditions in the brain that eventually cause the abnormal locomotor activities.

In conclusion, MPP^+ enhanced the methylation of phospholipids. The increment of PEMT activity by MPP^+ may help to explain MPTP neurotoxicity in causing parkinsonism and support a hypermethylation hypothesis in the pathogenesis of PD.

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