Proteasome-dependent degradation of cyclin D1 in 1-methyl-4-phenylpyridinium ion (MPP+) -induced cell cycle arrest

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Abstract

1-Methyl-4-phenylpyridinium ion (MPP⁺), an active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), induces cell death and inhibition of cell proliferation in various cells. However, the mechanism whereby MPP⁺ inhibits cell proliferation is still unclear. In this study, we found that MPP⁺ suppressed the proliferation with accumulation in G₁ phase without inducing cell death in p53-deficient MG63 osteosarcoma cells. MPP⁺ induced hypophosphorylation of retinoblastoma protein (Rb) and rapidly downregulated the protein but not mRNA levels of cyclin D1 in MG63 cells. The downregulation of cyclin D1 protein was suppressed by a proteasome inhibitor, MG132. The cyclin D1 downregulation by MPP⁺ was also observed in p53-positive PC12, HeLa S3 and HeLa ρ⁰ cells, which are a subclone of HeLa S3 lacking mitochondrial DNA. Moreover, MPP⁺ dephosphorylated Akt in PC12 cells, which was rescued by the pretreatment with nerve growth factor (NGF). In addition, the pretreatment with NGF or lithium chloride (LiCl), a glycogen synthase kinase-3β (GSK-3β) inhibitor, suppressed the cyclin D1 downregulation caused by MPP⁺. Our results demonstrate that MPP⁺ induces the cell cycle
arrest independently of its mitochondrial toxicity or the p53 status of the target cells, but through the proteasome- and phosphatidylinositol 3-kinase (PI3K)-Akt-GSK-3β-dependent cyclin D1 degradation.
Introduction

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is commonly used in animals to produce an experimental model of Parkinson Disease (PD) (1, 2). 1-Methyl-4-phenylpyridinium ion (MPP⁺) (3), an active metabolite of MPTP, induces cell death and inhibition of proliferation not only in neuronal cells but also in other type of cells (4-7). The mechanisms whereby MPP⁺ induces the inhibition of membrane-bound NADH dehydrogenase in mitochondrial complex I (8-10), ATP depletion (11), and production of the superoxide radicals (12, 13), have been extensively studied. However, the mechanisms involved in the inhibition of cell proliferation by MPP⁺ have rarely been examined.

Progression and transition in the cell cycle of mammalian cells are governed by cyclin-dependent kinases (Cdks) whose activities are regulated by the binding of their regulatory subunits (called cyclins) or Cdk inhibitors (CKIs) (14). p21 effectively inhibits Cdk2, Cdk3, Cdk4 and Cdk6, which have a direct role in the G₁/S transition, but it is a poor inhibitor of other known Cdks (15-17). Cyclin proteolysis is essential for cell cycle
progression (18, 19). Cyclin D1 regulates G₁ arrest and the phosphorylation status of retinoblastoma protein (Rb). Cyclin D1 degradation is mediated by phosphorylation-triggered, ubiquitin-dependent proteolysis (20). Cyclin D1 proteolysis is regulated by glycogen synthase kinase-3β (GSK-3β) (21), which is inactivated by a pathway that sequentially involves Ras, phosphatidylinositol 3-kinase (PI3K), and Akt (protein kinase B) (22).

The aim of this study is to examine the mechanisms whereby MPP⁺ inhibits cell proliferation. We show here that MPP⁺ initiates an efficient p53-independent G₁ arrest by the degradation of cyclin D1.

Methods:

Reagents and cells

MPP⁺ and MG132 were purchased from Sigma (St. Louis, MO) and Peptide Institute, Inc. (Osaka, Japan), respectively. Anti-cyclin D1 mouse monoclonal, anti-Rb mouse monoclonal, and anti-p21 rabbit polyclonal antibodies were purchased from BD
PharMingen (San Diego, CA). Anti-phospho-Akt (Ser473), anti-Akt, and anti-phospho-GSK-3β (Ser9) rabbit polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). MG63 and HeLa S3 were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin) at 37°C in a humid atmosphere containing 5% CO₂. PC12 cells were maintained in RPMI (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal horse serum (HS) and 5% FCS antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin) at 37°C in a humid atmosphere containing 5% CO₂.

Establishment of HeLa ρ⁰ Cells

As described by King and Attardi (23), HeLa S3 cells were maintained in αEU medium [αMEM(GIBCO) + 4 g/L glucose + 50 ng/ml etidium bromide (EtBr) + 0.1 g/L uridine] + 10% FCS for half a year. More than 100 colonies were isolated in αGU medium (αEU minus EtBr) + 10% FCS, of which the clone 9A (HeLa ρ⁰) cell strain was
selected by reason of its good growing potential and used in this study.

*Confirmation of mitochondrial DNA (mtDNA)*

The presence or absence of mitochondrial genome was confirmed in HeLa S3 and HeLa ρ^0^ cells with PCR using nine primer sets (24), which covered the whole mitochondrial genome. No amplification was shown with HeLa ρ^0^ DNA as template. Irregularly (once per 2-3 months), the absence of mtDNA in HeLa ρ^0^ cells has been confirmed using two primer sets. The sense primer is located at nucleotides (nt) 3108 to 3127 and the antisense primer at nt 3318 to 3301 of the published human mtDNA sequence (25). Product length was 211. Fragments of nuclear DNA were amplified with primers at nt 1437 to 1456 (GGCCATTGCAGAATTATTGC) and at nt 1530 to 1511 (AGCCATTCCAGCATATCGTC) in APC gene (product length was 94 bp). The PCR reaction mixture contained four primers (0.5 μM, each), 150 μM dNTPs, 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM MgCl₂, 0.1% TritonX-100, and 0.01% gelatin in a final volume of 10 μL. Ten nanograms of genomic DNA were submitted to PCR amplification (95°C for
30 seconds, 60\(^\circ\)C for 1 minutes, 72\(^\circ\)C for 1 minutes, 35 cycles) using 0.25 U of Taq polymerase (Takara, Tokyo).

**Western blot analysis**

Cells were collected and washed twice with ice-cold phosphate buffered saline (PBS), and then lysed with a solubilizing solution (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na\(_3\)VO\(_4\), 1 mM \(\beta\)-glycerolphosphate and 1 \(\mu\)g/ml leupeptin) on ice for 30 min. The extracts were cleared by centrifugation. Cell lysates were kept at 95\(^\circ\)C for 5 min and then separated by 12% (for cyclin D1 and p21), 10% (for Akt and GSK-3\(\beta\)), or 8% (for Rb) SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to the polyvinylidene difluoride membrane (Millipore Co., Bedford, MA). The membrane was treated with 10% (w/v) skim milk in PBS containing 0.05% Tween 20 overnight at 4\(^\circ\)C, and then incubated with primary antibodies, followed by peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amershan Pharmacia Biotech). The epitope was visualized with an ECL.
Western blot detection kit (Amershan Pharmacia Biotech).

*Cell cycle analysis by flow cytometry*

After the fixation with ethanol, the cells were treated with 50 μg/ml propidium iodide (Calbiochem) and analyzed by flow cytometer (FACSCalibur, Becton Dickinson) using CELLQUEST software.

*Northern blot analysis*

Total RNA was extracted using TRIzol reagent according to the manufacturer’s instructions (26). Ten micrograms of total RNA was electrophoresed, and transferred to Maximum strength Nytran nylon (Schleicher and Schrul, Knee, NH) with a Turbo-Blotter system (Schleicher and Schrul). The filter was hybridized with human cyclin D1 cDNA probe.
Results

**MPP⁺ suppresses the cell growth of p53-deficient MG63 cells**

Previous study showed that dopamine neurons from p53-knockout mice are resistant to MPTP neurotoxicity (27), suggesting that MPP⁺-induced apoptosis is at least partly mediated by p53 (28). To dissect the p53-independent effect of MPP⁺, we first examined whether MPP⁺ suppresses the growth of p53-deficient MG63 osteosarcoma cells. As shown in Fig. 1, MPP⁺ dose-dependently suppressed the cell proliferation. Under this condition, no appreciable cell death occurred by trypan blue exclusion test, which was confirmed by LDH release assay (data not shown). Thus, MPP⁺ inhibited the proliferation of MG63 cells without inducing cell death.

**MPP⁺ induces G₁ arrest in MG 63 cells.**

To examine the mechanism whereby MPP⁺ inhibits cell proliferation, the cell cycle was analyzed using a flow cytometer (FACS). The MG63 cells were incubated in DMEM with 0.5% FCS for 48 h to synchronize cells in G₀/G₁ (Fig. 2A). Then, these cells were
incubated in 10% FCS-supplemented medium in the absence or presence of 2 mM MPP⁺ for 16 h. The cell cycle analysis showed that MPP⁺ treatment induced accumulation in G₁ phase (Fig. 2C), whereas the control treatment markedly increased the population in S phase (Fig. 2B).

We next investigated whether the MPP⁺-induced G₁ arrest was accompanied by changes of Rb phosphorylation status (29). As expected, hypophosphorylated form of Rb increased after MPP⁺ treatment (Fig. 3A). However, MPP⁺ did not increase p21 protein level in p53-deficient MG63 cells (Fig. 3B), suggesting that MPP⁺ induces p53, p21-independent G₁ arrest in MG63 cells.

MPP⁺ downregulates cyclin D1 in a post-transcriptional mechanism

Cyclins are essential components of cell cycle machinery. During progression through the G₁ phase of the cell cycle, two major types of cyclins are required: D-type cyclins and cyclin E. Progression through G₁ depends initially on cyclin D-CDK4/6 protein complexes, and later on cyclin E-CDK2. D-type cyclins play a crucial role in the
progression through the cell cycle (30), and the downregulation of cyclin D1 plays an important role in the cell cycle arrest. Therefore, we focused on the change of cyclin D1 expression levels after MPP\(^+\) treatment. Although MPP\(^+\) suppressed the protein expression of cyclin D1 in dose-dependent and time-dependent manners (Fig.4A, B), MPP\(^+\) did not decrease the mRNA levels of cyclin D1 (Fig. 4C). These results indicate that the decrease of cyclin D1 levels is regulated by the posttranscriptional mechanism. Furthermore, MG132, a proteasome inhibitor, suppressed MPP\(^+\)-induced decrease in cyclin D1 protein level (Fig. 4D). These results indicate that MPP\(^+\)-induced downregulation of cyclin D1 is mediated by the proteasome-dependent signal pathway.

**MPP\(^+\) induces cyclin D1 downregulation even in the cells lacking mtDNA**

Next, we analyzed the effect of MPP\(^+\) on the level of cyclin D1 in a p53-positive neuronal cell line, PC12 and HeLa \(\rho^0\) cells lacking mtDNA. MPP\(^+\) downregulated cyclin D1 in PC12, HeLa S3, and HeLa \(\rho^0\) cells (Fig.5A, B, C). These results indicate that MPP\(^+\) downregulates cyclin D1 level in various cells and that its effect is independent of the p53
status or mitochondrial function in target cells.

**PI3K-Akt-GSK-3β pathway regulates MPP⁺-mediated cyclin D1 proteolysis**

Ubiquitin-proteasome-dependent degradation of cyclin D1 requires phosphorylation of the protein by GSK-3β, which is phosphorylated and inactivated by Akt (20). We therefore examined whether the PI3K-Akt-GSK-3β pathway is involved in MPP⁺-induced degradation of cyclin D1. Using PC12 cells, we demonstrated that MPP⁺ dephosphorylated Akt, and that the pretreatment with nerve growth factor (NGF) rescued the dephosphorylation (Fig. 6A). The phosphorylation of GSK-3β was augmented by the combined treatment with NGF and MPP⁺ (Fig. 6A). As shown in Fig. 6B, the pretreatment with NGF or lithium chloride (LiCl), an inhibitor of GSK-3β in cells (31, 32) suppressed the MPP⁺-induced degradation of cyclin D1. It was also confirmed in MG63 cells that LiCl but not KCl inhibited the cyclin D1 degradation caused by MPP⁺ (Fig. 6C). These results indicate that MPP⁺ induces the degradation of cyclin D1 through the PI3K-Akt-GSK-3β pathway.
Discussion

In the present study, we showed that MPP+ inhibits cell proliferation with accumulation in G, phase and hypophosphorylation of Rb in p53-deficient MG63 cells (Fig. 2C, 3A). In general, G, cell cycle arrest is induced by p53-dependent or p53-independent accumulation of p21, which inhibits cyclin-E/CDK2 activity and the G1-S transition (34, 35). Our results indicate that p53 is not associated with MPP+-induced growth inhibition. Although it has been reported that MPP+ inhibits the proliferation of PC12 cells by a p21-dependent pathway (6), MPP+ did not increase p21 protein levels in MG63 cells (Fig. 3B). These results suggest that MPP+ can inhibit cell proliferation independently of p53 or p21.

Cyclin proteolysis is essential for cell cycle progression (36). Inhibition of cyclin D1 expression either by antisense methodology or antibody microinjection lengthens the duration of the G, phase and suppresses cell proliferation (37, 38). Although cyclin D1 protein levels were rapidly downregulated by MPP+ within 4 h (Fig. 4B), the mRNA level of cyclin D1 did not decrease (Fig. 4C), suggesting that the downregulation of cyclin D1 is
regulated by the posttranscriptional mechanism. We showed that MPP⁺-induced decrease in cyclin D1 protein levels was suppressed by proteasome inhibitors, MG132 (Fig. 4D) or N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) (data not shown). These data suggest that MPP⁺ downregulates cyclin D1 through the ubiquitin-proteasome pathway (20).

MPP⁺ inhibits NADH dehydrogenase activity in mitochondrial complex I (8-10). However, we demonstrated that MPP⁺ downregulated cyclin D1 in HeLa and HeLa ρ⁰ cells (Fig. 5B, C), indicating that the downregulation of cyclin D1 is not associated with the inhibition of mitochondrial complex I by MPP⁺ and that MPP⁺ has an extramitochondrial molecular target. GSK-3β was reported to regulate cyclin D1 proteolysis (21). We showed that a GSK-3β specific inhibitor, LiCl, suppressed the MPP⁺-induced downregulation of cyclin D1 protein levels (Fig. 6B, C), suggesting the involvement of the GSK-3β pathway in the downregulation of cyclin D1. Akt inactivates GSK-3β through site-specific phosphorylation, resulting in the inhibition of cyclin D1 turnover (21). We demonstrated that MPP⁺ dephosphorylated Akt in PC12 cells and that the downregulation of cyclin D1 was suppressed by NGF, a well-known activator of the PI3K-Akt pathway or by an
inhibitor of GSK-3β, LiCl (Fig. 6A, B). The results indicate that MPP⁺ induces cyclin D1 degradation in various cell lines through the decreased Akt activity and the concomitant GSK-3β activation.

MPTP is a causative agent for the Parkinson model (1, 2). Since MPP⁺ is an active metabolite of MPTP (3), it is important to understand the molecular mechanism of MPP⁺ in vitro. Recently GSK-3β signaling has been implicated in neurodegeneration and apoptosis (39). The present study demonstrated that MPP⁺-induced G1 arrest results from the rapid degradation of cyclin D1 through the ubiquitin-proteasome- and PI3K-Akt-GSK-3β-dependent pathway, which indicates a possible new mechanism of MPP⁺-induced neurotoxicity.

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Figure legend

Fig. 1 MPP⁺ inhibits the proliferation of p53-deficient MG63 cells.

The equal number (2 x 10⁵) of MG63 cells were seeded on 10-cm dish and cultured with DMEM with 10% FCS for 16 h. Then cells were treated with the indicated concentration of MPP⁺ (0, 1, or 3 mM) in DMEM with 10% FCS for 0, 6, 12, and 24 h. The cells were trypsinized and the number of viable cells was counted by NucleoCounter (Chemometec, Denmark). The data are means ± SD of triplicate samples. * and ** denote p<0.05 and p<0.01, respectively, when compared with the sample without MPP⁺ treatment.

Fig. 2 MPP⁺ induces G₁ arrest in MG63 cells.

MG63 cells were incubated for 48 h in DMEM with 0.5% FBS to synchronize cells in G₀/G₁ (A). The synchronized MG63 cells were incubated in the medium with 10% FCS for 16 h in the absence (B) or presence of 2 mM MPP⁺ (C). Similar results in duplicates were obtained more than three times.
Fig. 3 MPP⁺ induces p53, p21-independent G1 arrest in MG63 cells.

MG63 cells were cultured with the medium containing 10% serum and 2 mM MPP⁺. After the indicated hours of culture, the cells were collected and subjected to Western blot analysis of phosphorylated Rb (A) and p21 (B). pp-Rb and p-Rb indicate hyperphosphorylated and hypophosphorylated form of Rb, respectively. The sample loading was monitored by staining with Coomassie brilliant blue R-250 (CBB). Similar results were obtained twice.

Fig. 4 MPP⁺ downregulates cyclin D1 in a posttranscriptional mechanism.

A, MG63 cells were cultured with the indicated concentration of MPP⁺ for 24 h. MPP⁺ decreased cyclin D1 protein levels in a dose-dependent manner. B, MG63 cells were cultured with 2 mM MPP⁺ for the indicated hours. MPP⁺ decreased cyclin D1 protein levels in a time-dependent manner. Similar results were obtained three times. C, 2 mM MPP⁺ did not decrease mRNA levels of cyclin D1 at the indicated time points by Northern blotting. Similar results were obtained three times. D, Cells were cultured with 2 mM MPP⁺ in the
absence or presence of a proteasome inhibitor, MG132 for 2 or 4 h. MG132 suppressed MPP⁺-induced decrease in cyclin D1 protein level. Similar results were obtained more than three times. The sample loading was monitored by CBB staining (A, B, D) or by 18S and 28S levels (C).

Fig. 5 MPP⁺ downregulates cyclin D1 in various cell lines.

The protein levels of cyclin D1 decreased when cells were cultured with MPP⁺. A, PC12 cells were cultured with 1 mM MPP⁺ for the indicated hours. B and C, HeLa S3 (B) and HeLa ρ⁰ cells (C) were cultured with or without 2 mM MPP⁺ for 4 h. The sample loading was monitored by CBB staining. Similar results were obtained twice.

Fig. 6 MPP⁺ downregulates cyclin D1 through the Akt-GSK-3β pathway

A, PC12 cells were pretreated with 50 ng/ml NGF for 30 min, and next treated with or without 1 mM MPP⁺ for 30 min. The expression levels of phosphorylated GSK-3β at Ser9 (P-GSK-3β), total and phosphorylated (at Ser 473) Akt (Akt and P-Akt, respectively) were
detected by Western blot analysis. Similar results were obtained in two additional experiments. B. PC12 cells were pretreated with 50 ng/ml NGF or 20 mM LiCl for 30 min, and next cultured with 1 mM MPP⁺ for 4 h. C, MG63 cells were pretreated with 20 mM LiCl or 20 mM KCl for 30 min, and next cultured with 2 mM MPP⁺ for 5 h. Cyclin D1 levels in cells were examined by Western blot analysis. The sample loading was monitored by CBB staining (B, C).
Fig. 1
Fig. 2

A: G1:70.2% S:17.8% G2:12.0%

B: G1:17.6% S:67.7% G2:14.7%

C: G1:80.8% S:6.3% G2:12.5%
Fig. 3

A

pp-Rb
p-Rb

CBB

Time (h): 0 2 4 8 24

B

p21

CBB

Time (h): 0 8 12 16 24
Fig. 5

A

Cyclin D1 →

CBB →

Time (h) : 0 1 2 4 6

B

Cyclin D1 →

CBB →

MPP⁺ (2 mM) : − +
Time (h) : 4 4

C

Cyclin D1 →

CBB →

MPP⁺ (2 mM) : − +
Time (h) : 4 4
Fig. 6

A

P-GSK-3β

P-Akt

Akt

NGF (50 ng/ml) : – + – + +
MPP⁺ (1 mM) : – – + + +

B

Cyclin D1

CBB

MPP⁺ (1 mM) : – + + – + –
LiCl (20 mM) : – – + + – –
KCl (20 mM) : – – – – + +

C

Cyclin D1

CBB

MPP⁺ (2 mM) : – + + – + –
LiCl (20 mM) : – – + + – –
KCl (20 mM) : – – – – + +
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