Protective effect of valproic acid on MPP⁺-induced neurotoxicity in dopaminergic SH-SY5Y cells through Cdk5/p35/Erk signaling cascade

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Abstract

Purpose: To investigate the neuroprotective effect of valproic acid (VPA) on 1-methyl-4-phenylpyridinium (MPP⁺)-induced dopaminergic cell loss in human neuroblastoma SH-SY5Y cells.

Methods: Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Reactive oxygen species (ROS) generation in MPP⁺-treated SH-SY5Y cells was investigated by DCFH-DA. Apoptotic cell death was confirmed by Hoechst 33342 staining. The protective effect of VPA via Cdk5 and p35 cascade was investigated by reverse transcription polymerase chain reaction (RT-PCR) and Western blot (WB) analysis. In addition, further investigation on cell survival was performed using Western blot analysis through Erk signaling pathway.

Results: Cell viability was dramatically decreased in cells treated with MPP⁺ in a concentration-dependent manner (p < 0.05). Pre-treatment with VPA ameliorated MPP⁺-induced death of dopaminergic cell via inhibition of ROS generation (p < 0.05). VPA restored Cdk5 and p35 expression and significantly increased cell survival mediated by Erk activity (pErk/Erk).

Conclusion: The results from this study confirmed that VPA attenuated MPP⁺-induced dopaminergic cell death by the inhibition of ROS production via Cdk5/p35 cascade and Erk signaling pathway. VPA is thus a potential therapeutic candidate for the treatment of dopaminergic cell death via Cdk5/p35 cascade.

Keyword: Valproic acid, Neuronal cells, Cyclin-dependent kinase 5, p35, Erk signaling pathway
INTRODUCTION

Parkinson’s disease (PD) is a progressive disorder that affects dopaminergic neuron loss in the substantia nigra (SN) pars compacta. The loss of tyrosine hydroxylase (TH) contributed to dopamine (DA) deficiency observed in PD [1]. Although the cause and the mechanism of neuronal cell death in PD are unknown, oxidative stress could be one of the factors involved with PD pathogenesis. A previous study investigated the mechanism of PD via using anti-oxidative agent [2] and in another study, the level of reactive oxygen species (ROS) was significantly increased in association with dopaminergic neuronal cell death by 1-methyl-4-phenylpyridinium (MPP⁺), the toxic metabolite of neurotoxic agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [3]. MPP⁺-induced dopaminergic cell death occurred via several signaling cascades, including PI3K/Akt/GSK3β via Erk pathway [5]. Recent evidence demonstrated that cyclin-dependent kinase 5 (Cdk5) plays an important role in neural development and neurodegenerative diseases [6] and Cdk5 hyperactivated by oxidative stress was observed in neurodegenerative disease [7]. Cdk5, unlike other members of the Cdk family, is activated by co-activator p35. The level of p35 protein was reported to be regulated via the kinase activity of Cdk5 [8] and expression of Cdk5 and its activator, p35, were involved with the induction of apoptosis in SN neurons [9].

Valproic acid (VPA) is widely used in epilepsy treatments. Recently, the neuroprotective effects of VPA have been revealed in dopaminergic cell death [10], however, the mechanism is still unclear. Therefore, this study aims to investigate the protective effect of VPA on MPP⁺-induced dopaminergic cell death. A better understanding of the mechanism of the survival signaling pathway in SH-SY5Y cell line was also obtained.

EXPERIMENTAL

Cell culture

SH-SY5Y cells were purchased from ATCC (Bethesda, MD, USA) and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin (GIBCO, Grand Island, NY, USA). Cells were incubated at 37 °C under 5% CO₂ in a humidified atmosphere.

Cell viability assay

SH-SY5Y cells were seeded in a 96-well culture plate at 1.5 x 10⁴ cells per well for 24 h. Cells were pretreated with VPA for 1 h before 24 h incubation with MPP⁺. Cells were then incubated with MTT (Sigma Chemical CO, USA) for 4 h. After DMSO was added, the optical densities were read at 540 nm. Cell viability was reported as a percentage ratio in comparison to the untreated control groups.

Hoechst 33342 staining

After MPP⁺ treatment for 24 h, with or without pre-treatment with VPA, cells were stained with 10 μg/ml Hoechst 33342 (Sigma Chemical CO, USA) for 30 min at room temperature in the dark. The nuclei were visualized with a fluorescence microscope (Olympus, Japan). Condensed chromatins and fragments of nuclei were considered apoptotic cells. The number of apoptotic cells were scored and calculated related to the total number of nuclei.

Production of intracellular reactive oxygen species (ROS)

Intracellular ROS production was measured by DCFH-DA (Sigma Chemical CO, USA). Cells were seeded at 5x10⁴ cells per well and pretreated with VPA. The pretreated cells were exposed to MPP⁺ for 24 h prior to incubation with DCFH-DA dye for 1 h, followed by washing with PBS. Fluorescence intensity was then measured using a fluorescence microplate reader (Bio-tex, USA) at 485 nm and 530 nm for excitation and emission, respectively.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from treated and untreated cell cultures using Trizol® reagent (Invitrogen, USA) according to the manufacturer’s instructions. cDNA was generated for RT-PCR using the SuperScript® III First-Strand Synthesis System using the primer pairs shown in Table 1.

Table 1: Sequence of RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdk5</td>
<td>5'-GGCACTACGGAAACTGTGT-3'</td>
<td>5'-CTCAGGATCGAGGTCCACCAT-3'</td>
</tr>
<tr>
<td>p35</td>
<td>5'-ACG GTCTGTCCCTGCT-3'</td>
<td>5'-TGCCGTCTTCTTGTGTGT-3'</td>
</tr>
<tr>
<td>TH</td>
<td>5'-AGTAGCACCAC GTATATCGCC-3'</td>
<td>5'-GAACCTCCACGTAACGATG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-ACCCACAGTCC ATGCACTAC-3'</td>
<td>5'-TCCACCCACTGTGTGCTGA-3'</td>
</tr>
</tbody>
</table>
All reactions were in triplicate. The signal optical densities were quantified using ImageJ analysis software (NIMH, USA). Gene expression levels were then calculated based on the relative density of GAPDH housekeeping gene.

**Western blot**

Total proteins extraction was performed using RIPA buffer. Protein concentration determination was measured using the Bio-Rad protein assay (Hercules, CA, USA). The same amount of protein was subjected to SDS-PAGE. The proteins were transferred to PVDF membrane (Millipore, USA). The blotting membrane was incubated in 5 % skimmed milk (w/v) and further incubated with primary antibodies.

HRP-linked antibodies (Abcam) were applied as secondary antibodies. The signals were developed with SuperSignal West Pico chemiluminescence substrate and the signal optical densities were quantified using ImageJ analysis software (NIMH, USA).

**Statistical analysis**

Data are expressed as mean ± SEM (n = 3). Data analysis was performed by one-way ANOVA, followed by two-tailed Student’s t test using SPSS version 16.0 (SPSS, Cary, NC, USA). Significance level was defined as p < 0.05.

**RESULTS**

**VPA ameliorates MPP⁺-induced loss of neuronal cell viability**

MPP⁺ inhibited cell viability in a concentration-dependent manner in the range of 10-1000 µM (Figure 1A). The viability of cells pretreated with 100 µM VPA for 1 h prior to exposure to MPP⁺ was greater than the viability of untreated exposed cells (Figure 1B). The reduction of MPP⁺-induced cell viability loss indicated a possible protective effect of VPA. Hoechst 33342 staining results (Figure 1C) confirmed the protective effect of VPA against MPP⁺-induced cell death.

A significant presence of DNA fragmentation (indicated as arrows in Figure 1C) was apparent in untreated cells exposed to 100 µM MPP⁺ while the presence of apoptotic cells in pretreated exposed cells was significantly decreased. The percentage of apoptotic cells was then quantified (Figure 1D).

**VPA suppressed MPP⁺-induced oxidative stress**

As shown in Figure 2A, treatment with 100 µM MPP⁺ for 3, 6, 9, 18 and 24 h significantly produced intracellular ROS when compared to the untreated control. The highest level of ROS in MPP⁺-treated cells was observed at 3 h. Cells pretreated with VPA significantly decreased intracellular ROS compared to MPP⁺-treated cells (Figure 2B).

**Effect of VPA on gene expression levels of TH, Cdk5 and p35 in cells exposed to MPP⁺**

The expression level of TH mRNA was significantly decreased in the cells exposed to MPP⁺, while the expression level of Cdk5 and p35 mRNA were significantly increased. Cells pretreated with VPA restored Cdk5, p35 and TH mRNA level when compared to the MPP⁺-treated cells (Figure 3).

**Effect of VPA on protein expressions of TH dopaminergic neuronal marker, Cdk5 and p35 in MPP⁺-induced cell death**

Cells treated with 100 µM of MPP⁺ for 24 h showed a significant decrease of TH protein levels while the protein expression of Cdk5 and p35 was significantly increased. This result corresponded with the gene expression experiment. Pre-treatment with VPA significantly
restored protein levels of Cdk5 and p35 when compared to the MPP⁺-treated cells (Figure 4).

![Figure 2](image1.png)

**Figure 2**: VPA suppressed MPP⁺-induced oxidative stress. (A) Time-dependent ROS levels produced by cells incubated with 100 µM MPP⁺. (B) Cells were pretreated with 100 µM VPA for 1 h prior to incubation with or without 100 µM MPP⁺ for 3 h. DCFH-DA was used for detecting ROS generation. All values were presented as mean ± SEM; *p < 0.05 versus control untreated group; #p < 0.05 versus MPP⁺-treated group

VPA protected MPP⁺-induced dopaminergic neuron loss via Erk signaling pathway

To examine whether VPA could ameliorate MPP⁺-induced dopaminergic neuron loss or not, the expression of Erk and phosphorylation of Erk (p-Erk) were determined. As shown in Figure 5, MPP⁺-treated cells significantly decreased p-Erk/Erk activity in the control group. However, in the pre-treatment with VPA, cells showed a significant increase in p-Erk/Erk activity, in comparison to MPP⁺-treated cells.

**Figure 3**: Effect of VPA on the mRNA expression level of Cdk5, p35 and TH during MPP⁺-induced dopaminergic neuron death. SH-SY5Y cells were pretreated with 100 µM VPA with or without treatment with 100 µM MPP⁺. Expression levels were presented as mean ± SEM; *p < 0.05 versus control untreated group; #p < 0.05 versus MPP⁺-treated group

**Figure 4**: Effect of VPA on the protein expression level of Cdk5, p35 and TH in MPP⁺-induced dopaminergic neuron death. (A) WB analysis of SH-SY5Y cells pretreated with 100 µM VPA with or without treatment with 100 µM MPP⁺. (B) Bands were quantified by Image J. All values were represented as mean ± SEM; *p < 0.05 versus control untreated group; #p < 0.05 versus MPP⁺-treated group

**DISCUSSION**

PD causes dopaminergic cell loss in the SN [11]. SH-SY5Y cells have been reported to express dopaminergic neuronal markers and served as a well-established PD model [12]. MPP⁺ is also a widely used model for studying the pathogenesis of PD [3]. However, the mechanism involved in dopaminergic cell loss in PD is still not well understood. In the present study, we provide the protective evidence of VPA on MPP⁺-induced dopaminergic cell loss in SH-SY5Y cells. The mechanisms involved with the protection of dopaminergic cell loss were also investigated.

MPP⁺ was able to induce ROS production and
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Figure 5: VPA protected MPP⁺-induced dopaminergic neuron loss via Erk signaling pathway. (A) SH-SY5Y cells pretreated with 100 µM VPA with or without treatment with 100 µM MPP⁺. (B) The protein bands were quantified by Image J. All values were presented as mean ± SEM; *p < 0.05 versus control untreated group; #p < 0.05 versus MPP⁺-treated group

led to the loss of nigrostriatal neurons in PD [13] and a significant reduction of TH positive neurons after MPP⁺ treatment was also reported [14]. Examination of SN cells from PD-affected human brain tissue showed oxidative damage of DNA and protein [15]. In consistent with the previous studies, cells exposed for 24 h to MPP⁺ revealed an increased level of ROS production and induced toxicity in dopaminergic neurons. The excessive level of ROS could then damage mitochondria, leading to the hyperactivation of Cdk5-induced cell death [16]. Increased levels of Cdk5 protein were also observed in the brains of PD patients [17]. In this study, high levels of both mRNA and protein expressions of p35 and Cdk5 were detected in MPP⁺-treated cells compared with the control. The study by Town et al [18] showed that the p35/Cdk5 pathway is associated with neurodegenerative disease. Taken together, our data suggested an association between Cdk5/p35 and ROS in the MPP⁺-induced dopaminergic neuronal death. However, the association between ROS and Cdk5 needs to be further investigated.

VPA has recently been used as neuroprotective drug in the therapy of several neurological diseases, including PD, and also acted as a potent histone deacetylase (HDAC) inhibitor [19]. Neurotoxicity was increased in cells with high Cdk5 activity and suppressed HDAC function, which lead to an increase of DNA damage and neuronal cell death [20]. VPA, however, downregulated the Cdk5 activity in cells by reducing expression of p35 mRNA [21]. Acute and chronic treatments with VPA were also reported to affect TH mRNA expression levels in rat locus coeruleus [22]. The results of the present study also indicated that VPA could attenuate MPP⁺-induced cell death by lowering the level of p35 and Cdk5 mRNA and protein expression.

VPA attenuated α-synuclein toxicity via the Erk pathway. Erk was reported previously to play a pivotal role in neuronal survival in PD model [23]. Cell proliferation was also induced through the Erk pathway and mediated induction of p35 [24]. However, the links between VPA and dopaminergic cell loss through Erk pathway has remained obscure. In this study, the experimental data supported that MPP⁺ could reduce Erk activity but pre-treatment of cells with VPA gave the reverse the effect. The WB analysis of cells pretreated with VPA confirmed the inhibition of MPP⁺-induced dopaminergic cell loss through the regulation of Cdk5 and Erk protein expression. Cdk5, thus, regulates TH activity through Erk [15]. Taken together, our results indicated that VPA attenuated dopaminergic cell death by decreasing ROS generation and restoring Cdk5 and p35 levels via the Erk pathway.

CONCLUSION

The findings of this study demonstrate that pre-treatment with VPA exerts a positive effect on cell survival of MPP⁺-treated dopaminergic SH-SY5Y cells. The results also reveal that VPA protects against dopaminergic neuronal death via Cdk5/p35 and the Erk signaling pathways. Thus VPA can be potentially used as a candidate therapeutic drug based on the inhibition of dopaminergic cell death through the generation of ROS, the activity of Cdk5 and the Erk signaling pathway.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jakkapong Muangsab and Piliawanwadee Hutamekalin designed the experiments, performed data analysis and statistics, and drafted the manuscript. Peerada Prommeenate, Banthit Chetsawang, Pennapa Chonpathompikunlert and Wanida Sukketsiri provided professional comments for this work and helped with the data analysis. All authors read and approved the final manuscript.

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