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Voltage-Gated potassium channel KV1.5 protects against MPP^+ mediated neurotoxicity in PC12 cells

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Abstract: Background: Parkinson's disease (PD) is the second most common neurodegenerative disease and afflicts almost 1.8% of over 65-year-old group in the world. Epidemiological projections showed that the incidence of PD was increasing continuously each year, with a wider age range as well. A large number of studies indicated that voltage-gated potassium channel (Kv) played significant roles in cellular signaling in both excitable and non-excitable cells. What's more, Kv was also ubiquitously expressed in neurons and participated in signaling pathway in neurons. Kv1.5 (encoded by KCNA5) is an important voltage-gated K^+ channel, which is not only necessary for critical processes such as cell proliferation and apoptosis but ubiquitously expressed in neurons. Recent studies reported that PD clinical drugs could inhibit the expression of Kv1.5. To determine the mechanisms by which Kv1.5 protects against MPP^+ mediated neurotoxicity in PC12 cells. Materials and Methods: Knockdown of Kv1.5 model was established with pSINsi-hU6- Kv1.5 treated by the RNAi method in PC12. MTT, and Western Blot were used to detect the influence of Kv1.5 on PC12 proliferation, and the effect of Kv1.5 on PC12 apoptosis after MPP^+ treatment *in vitro*. Results: 1) Knockdown and overexpression of Kv1.5 participated in PC12 proliferation. Transiently over-expressed Kv1.5 could boost the survival rate of PC12, while transiently knockdown of Kv1.5 inhibited PC12 proliferation. 2) The effect of Kv1.5 on PC12 proliferation was through PI3K/Akt signaling pathway. Over-expressed Kv1.5 could induce the activation of Akt, and Bcl-2 expression in PC12; Knockdown of Kv1.5 in PC12 inhibited the activation of Akt, Bcl-2 expression, and promoted MAPK phosphorylation. 3) Over-expressed Kv1.5 could significantly prevent PC12 from apoptosis induced by MPP^+ via activating Akt pathway and increasing Bcl-2 expression; Knockdown of Kv1.5 was more sensitive than its control counterpart when treated with MPP^+ for 24 h. Conclusion: Kv1.5 could hinder MPP^+ neurotoxicity to PC12 by PI3K/Akt signaling pathway.

Keywords: PC12, Kv1.5, MPP^+, PD, PI3K/Akt

1. Introduction

Neuronal protection or prevention of neuronal degeneration has been identified as therapeutic targets for many neurodegenerative diseases [1]. Parkinson's disease (PD) is the second most common neurodegenerative diseases affecting about 1-2% of the population over the age of 65 years, and afflicting over 6 million people worldwide [2]. Postmortem studies have showed the involvement of several predisposing factors including viral infection and environmental toxins for example MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine). MPTP mediated selective damage to dopaminergic neurons of nigrostriatal pathway has been widely used as a model of PD [3]. MPTP requires monoamine oxidase B to be converted into MPP^+. MPP^+ is selectively taken up by dopaminergic neurons and cause PD [4]. PC12 cells, a rat clonal pheochromocytoma cell line, possess dopamine synthesis, metabolism and transporter systems therefore have been used as a model for studies of MPP^+ neurotoxicity and PD [5]. Some manifestations of

neurodegenerative diseases likely reflect alterations in the function of neurons rather than neuronal atrophy and loss. Electrophysiological changes accompanying neuronal dysfunction, in the case of Huntington's disease, it has been suggested that initial stages of the neurodegenerative result from dysfunction rather than cells death [6]. It is not surprising that K^+ channels comprise the largest and most diverse class of ion channels which may be sub-divided into voltage gated, Ca^{2+} dependent, 2-pore domain, and inward rectifier K^+ channels [7]. K^+ channels play an important role in setting the resting membrane potential and determining repolarization in the functionally diverse systems. They also affect mitotic cell cycling, proliferation, and development of cancer [8]. Jankovic (1985) reported that Parkinson's disease patients total body potassium have high levels [9], Sutton(2012) suggested that elevated K^+ may provide an ionic mechanism that can contribute to the therapeutic of advanced PD[10]. Neuronal Kv channels might be a potential target for the development of therapeutic strategies for some neurodegenerative disorders [11]. Kv1.5 (KCNA5) is expressed in the heart, where it underlies the I (Kur) current that controls atrial repolarization, and in the pulmonary vasculature [12]. Moreover, Kv1.5 is important for critical processes such as cell proliferation and apoptosis [13, 14], which indicates that Kv1.5 could have significance in the survival and function of neuronal cells. Besides Kv1.5 is also ubiquitously expressed, including many regions of the brain such as cerebral cortex, hippocampus, gliocyte, pituicyte [15, 16].

In this study, we investigated the molecular mechanisms underlying Kv1.5 in PD and reveal the involvement of the PI3K/Akt signaling pathway in the MPP^+ induced PC12 apoptotic.

2. Results

2.1. Successful Model that MPP^+ Induced PC12 Apoptotic Was Established

To examine the toxicity of MPP^+ on PC12, we treated cells with MPP^+ at different concentration (200 μM, 500 μM, 1 mM) for 24 h. MTT assay the cells survival rate. As shown in figure 1a, the cells proliferation was inhibited at 500 μM, 1 mM, but not at 200 μM. Western blot analysis with a phospho-specifc Akt antibody was performed to assess the phosphorylation levels of Akt. As shown in figure 1b, we found that MPP^+ was able to decrease the activation of Akt at 500 μM.

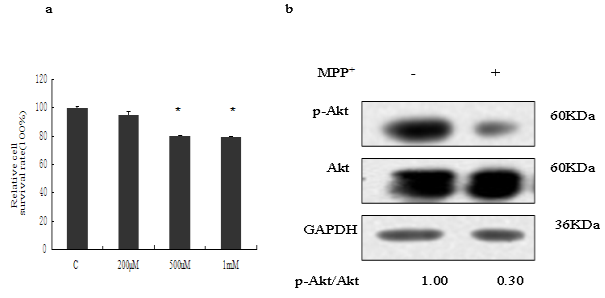

Figure 1. *Established PC12 cells apoptotic model with MPP^+.*

(a) Neuronal PC12 cells were treated with 200 μM, 500 μM, 1 mM MPP^+ for 24 h. Cell proliferation was inhibited by 500μM and 1mM. (b) Cell extracts were subjected to SDS-PAGE and Western immunoblotting. Proteins were probed with antibodies for p-Akt and total Akt as loading control. (n=4, $^{**}P<0.01$, $^{*}P<0.05$).

2.2. The effect of Kv1.5 on PC12 Survival

Kv1.5 has been shown to play a key role in cell proliferation and apoptosis. Thus we anticipated that perhaps Kv1.5 would be necessary for the survival of PC12. PC12 were transiently over-expressed, Western blot assayed the protein expression of Kv1.5 in PC12, as indicated in figure 2a, Kv1.5 level was increased after over-expressed Kv1.5. In order to elucidate the effect of suppressed Kv1.5 in PC12, siRNA method was employed. As shown in figure 2b, we assayed the protein expression, illustrated that the level of Kv1.5 in PC12-SK was lower than control cells. To confirm our hypothesis we assayed viability (using MTT assay) on control and Kv1.5 overexpression cells, as shown in figure 2c, 2d, overexpression Kv1.5 increase survival growth rate. Knockdown of Kv1.5 gene decreased PC12 relative survival rate. The effect of Kv1.5 on the PC12 survival-signaling pathway was assayed by assessing the anti-apoptotic protein p-Akt. Akt serine/threonine kinase activity is primarily activated by phosphorylation through PI3K. Here, we measured the activity of Akt by means of Western blotting. As shown in figure 2e, we found an observably increase in p-Akt levels in Kv1.5 overexpression PC12. It's well known that Bcl-2 up-regulation/stabilization was through PI3K/Akt activation, thus we determined that the levels of the anti-apoptotic protein Bcl-2 in different expression of Kv1.5 in PC12. As shown in figure 2f Bcl-2 was increase in Kv1.5 overexpression PC12.

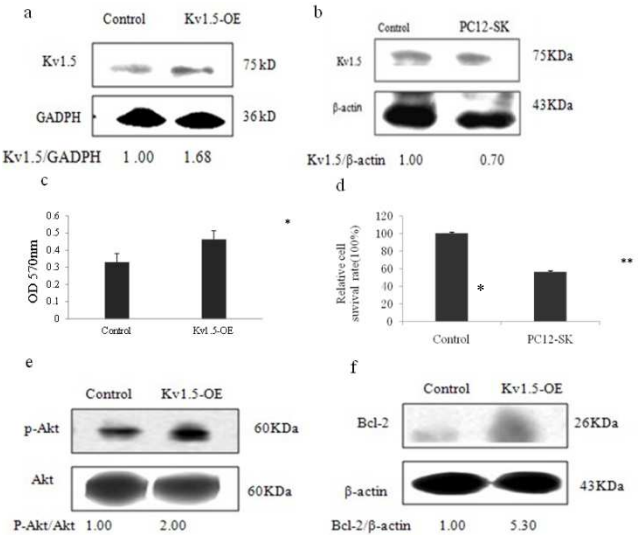

Figure 2. *The effect of Kv1.5 on PC12 survival*

Neuronal PC12 cells were transfected with overexpression Kv1.5 or Kv1.5 siRNA plasmid and after 48 h. (a) Cells extracts were subjected to SDS-PAGE and Western

immunoblotting. Proteins were probed with antibodies for Kv1.5 and GAPDH as loading control. (b) Proteins were probed with antibodies for Kv1.5 and β-actin as loading control. (c) and (d) MTT assayed cells proliferation. (e) Proteins were probed with antibodies for p-Akt and Akt as loading control. (f) Proteins were probed with antibodies for Bcl-2 and β-actin as loading control (n=4, $^{**}P<0.01$, $^{*}P<0.05$).

2.3. The effect of Kv1.5 Overexpression or Knockdown on MPP$^+$ Induce PC12 Apoptotic

To examine the effect of Kv1.5 role in MPP^+ toxicity, we transiently over-expressed Kv1.5 in PC12. PC12 was treated with determined 500 μM MPP^+ for 24 h, showed a significant decrease in cells survival as compared with control untreated cells. However, MPP^+ didn't affect the survival of overexpression Kv1.5 in PC12 (3a). When PC12-SK was treated with MPP^+ for 48 h, PC12-SK was significantly more sensitive than control cells (3b).

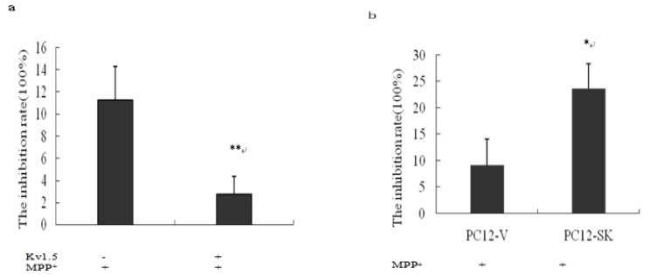

Figure 3. *The effect of Kv1.5 overexpression or knockdown on MPP^+ induce PC12 apoptotic*

Neuronal PC12 cells were transfected with over-expressed Kv1.5 or Kv1.5 siRNA and after 24 h, were treated with 500 μM MPP^+ for 24 h. (a, b) MTT assayed the cells inhibition rate (n=3, $^{**}P<0.01$, $^{*}P<0.05$).

2.4. Kv1.5 inhibits MPP$^+$ Induced Toxicity through PI3K/Akt/Bcl-2 Pathway

To confirm that Kv1.5 protects the cells against MPP^+ mediated death through activation of the PI3K/Akt survival pathway. PC12 and overexpression Kv1.5 was treated with MPP^+ for 24 h. As depicted in figure 4a, MPP^+ inhibited phosphorylation of Akt was more in PC12 than overexpression Kv1.5 in PC12 cells. When 4-AP treated PC12 cells overexpression Kv1.5 that treated with MPP^+, activation of Akt was decrease. MPP^+ inhibited Akt activation, and MPP^+ inhibited phosphorylation of Akt was more significant in Kv1.5 silence PC12 than control (4b). Bcl-2 was protected from MPP^+-induced cells death. We wanted to know whether Bcl-2 was also involved in Kv1.5 mediated neuroprotection in PC12. Bcl-2 level was lower in Kv1.5 gene knockdown cells as compared with control (4c). After cells were treated with 500 μM MPP^+, inhibited MAPK activation, However, MPP^+ inhibited phosphorylation of MAPK was lower in Kv1.5 gene knockdown PC12 cells than control (4d). Based on the affinity of 4-AP for block of various potassium channels subtypes and the expression pattern of channels in the PC12. The most likely target of therapeutic concentrations of 4-AP were the Kv1 family of potassium channels, specifically the Kv1.5 channel which have the highest affinity for 4-AP[17]. Alviña and Khodakhah reported that 10 μM 4-AP most likely block Kv1.5 potassium channels. Therefore we tested whether 4-AP participate in MPP^+ neurotoxicity in PC12. 200 μM MPP^+ didn't mediate PC12 cells apoptosis (1a), however, PC12 were treated with 10 μM 4-AP and 200 μM MPP^+ significance inhibited cells proliferation (4e). As shown in figure 4f, 10 μM 4-AP could signally inhibited Akt activation.

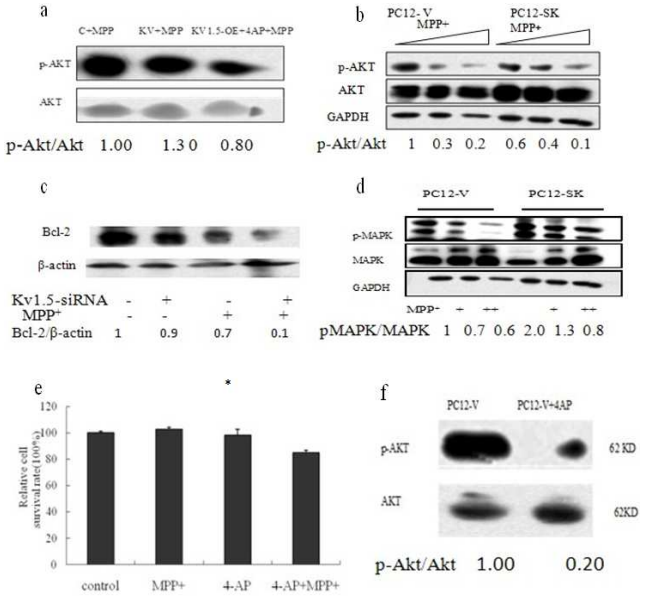

Figure 4. *Kv1.5 inhibits MPP^+ induced toxicity through PI3K/Akt/Bcl-2 pathway*

Neuronal PC12 cells were transfected with over-expressed Kv1.5 or Kv1.5 siRNA, and after 24 h were treated with 500 μM MPP^+ for 24 h. (a) Relative activated of p-Akt in control, over-expression Kv1.5, and after over-expression Kv1.5 treated with 4-AP Akt as loading control. (b) Proteins were probed with antibodies for p-Akt and Akt as loading control. (c) Proteins were probed with antibodies for Bcl-2 and β-actin as loading control. (d) Proteins were probed with antibodies for p-MAPK and MAPK as loading control. (e) Neuronal PC12 cells were exposed to 200 μM MPP^+ for 24 h, and treated with or without 4-AP for 24 h. MTT assayed the cells proliferation. (f) Neuronal PC12 cells were treated with 4-AP for 24 h. Proteins were probed with antibodies for p-Akt and Akt as loading control (n=4, $^{*}P<0.05$).

3. Discussion

Parkinson's disease (PD) is one of the most common neurodegenerative disorders and still remains incurable and can be triggered by genetic or environmental factors [18, 19]. Metal ions dyshomeostasis appears to play a key pathogenic role in a number of these maladies, including the most common of neurodegenerative diseases (Alzheimer's disease,

Parkinson's disease) [20]. Moreover, these metal ions may induce chemical modifications on the protein *in vitro* and in the reducing intracellular environment [21].

A large number studies indicate that potassium (K^+) channels play important roles in cellular signaling in both excitable and non-excitable cells. Moreover, a considerable number of K^+ channels within the nervous system appear to mediate diverse cellular signaling, including regulation of neurotransmitter release, neuronal excitability, and cell volume. Although the precise etiopathogenesis of the PD remains unknown, recent studies on the K^+ channels gene expression in the basal ganglia reveal dysfunctions of various K^+ channels, including Kv, K_{ATP}, Kir, SK, and K_{2P} channels, etc, which may be involved in the pathogenesis of Parkinson's disease[22]. 2005, Hong reported that pergolide produces clinical benefit in Parkinson disease, however, is an inhibitor of voltage-gated potassium channels, including Kv1.5. Thus Kv1.5 could play an important role in PD[23]. However, the mechanisms that underlie the protective effect of Kv1.5 on neuronal degeneration especially in PD have not been identified.

Most studies were devoted to identify the impact of Kv1.5 channel on the proliferation of tumor cells[24, 25]. Moreover, the importance of K^+ in cell survival is supported by our result. First, up-regulation of Kv1.5 increases cells proliferation and restricts apoptosis in PC12. Second, down-regulation or inhibition of Kv1.5 channel resulted in inhibiting survival by PI3K/Akt signaling. We also have elucidated the role of Kv1.5 in degeneration of dopaminergic neurons. Our results demonstrate that the transiently overexpression of Kv1.5 increase PC12 survival, and activated Akt, and transiently knockdown of Kv1.5 inhibited PC12 survival. Stably knockdown of Kv1.5 didn't have significance in PC12 survival. Knockdown of Kv1.5 would inhibit the outward potassium current and lead to an increased cells membrane permeability. At the same time, the exchanging rate between cells and external environment would be reduced. As a result, knockdown of Kv1.5 finally inhibited the cells proliferation. Recent research showed that some Kv channels reduced while other ion channels over-expressed. For example, Kv1.5 was over-expressed while Kv1.3 expression reduced in brain cancer. What's more, potassium channels' functions also are not the same. So we inferred that after knockdown Kv1.5 in PC12, some potassium channels were over expression in order to maintain homeostasis.

MPP^+-induced neurotoxicity is an established model of PD, also in neuronal differentiated PC12[26, 27], a system we used in the present studies. The systemic administration of chronic MPP^+ led to the death of dopaminergic neurons. Our results demonstrate that the overexpression of Kv1.5 didn't significant inhibited survival after MPP^+ treatment in PC12, Kv1.5 gene knockdown was more sensitive for MPP^+. Previously, accumulating evidence suggested that activation of the PI3K/Akt pathway inhibited MPP^+ induced PC12 apoptosis[28, 29]. The salient feature of the data presented in this report was the finding that Kv1.5 involved in MPP^+ induced neurotoxicity. Kv1.5 inhibited MPP^+ induced PC12 apoptosis by activation of the PI3K/Akt survival pathway, increased Bcl-2.

Our cells studies showed that overexpression of Kv1.5 increased Akt activation, and promote cells survival. Akt activation is known to be critical for neuronal anti-apoptosis. Knockdown of Kv1.5 decreased Akt activation and Bcl-2 expression in PC12. Overexpression of Kv1.5 showed a significant increase in membrane Kv1.5 levels, K^+ influx and consequently showed more resistance to MPP^+-induced cell death. PC12-SK was more sensitive than control. Based on these findings, we postulated that MPP^+-treatment would contribute to altered K^+ homeostasis thereby leading to cells death.

4. Materials and Methods

4.1. Chemicals and Antibodies

PC12 Cells purchased from shanghai cell culture center, MPP^+. PRIM1640 purchased from Hyclone (USA).The anti-Kv1.5 Antibody was purchased from Millipore, the anti-Bcl-2 antibody purchased from cell signaling, the anti-β-actin antibody purchased from Boster (China), the anti-p-Akt purchased from Cell Signaling Technology (USA), the anti-Akt and other antibodies all purchased from Santa Cruz Biotechnology (USA).

4.2. Cell Culture and Treatment and Growth Assay

All parental and derivative cells were maintained at 37°C in a 5% CO_2 atmosphere in PRIM1640 and 15% new calf serum in a humidified incubator. For MPP^+ treatment, cells were maintained in phenol red media with 15% new calf serum for 1 day. For Akt activation assays, cells were treated with vehicle (PBS) and indicated concentrations of MPP^+. To test the effects of 4-AP (4-aminopyridine) for MPP^+, the 4-AP was added at the same time with MPP^+ for 24 h. To examine cells growth in the presence or absence of MPP^+, cells maintained for 1 or 2 days in PRIM1640 and 15% new calf serum were treated with MPP^+ and PBS vehicle as a control. The cells were seeded at 1×10^4 cells per dish in 96-well plates dishes and the cell numbers were determined using the MTT after 2 days. Five dishes were used for each treatment and experiments were repeated >3 times.

Cell lines with Kv1.5 expression knocked down by the siRNA method in PC12 cells and PC12-SK cells were generated.

4.3. Plasmids, DNA Transfection

The Kv1.5 cDNA was from Dr. Zhengjie (University of California, Davis, USA). Cells were cultured in each well of the six-well plates at 90% convergence degree, and 4 μg siRNA was transfected into cells using Lipofectamine 2000 TM (Invitrogen) according to the manufacturer's instructions. Transient transfection cells were harvested in 48 h. Overexpression Kv1.5, in PC12 cells were performed using respective plasmid, cells were harvested for either Western blot and MTT assay.

4.4. Western Blotting

The normal cells were harvested in PBS and precipitated by centrifugation at 1000 $rmin^{-1}$, 4°C for 10 min. Cell pellets were washed twice with cooling PBS and then incubated on ice with the lysis buffer 20 mmol L^{-1} Tris-HCl, pH7.5, 70 mmol L^{-1} NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1% PMSF) for 1 h to extract the proteins. Protein concentration was determined according to Bradford. The transfected cells were lysed on ice with a 1×SDS loading buffer. Crude lysates were collected in an Eppendorf tube. Proteins were resolved by 10% SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, blocked in PBST (pH 7.4) containing 5% dried milk powder. After blocking, membranes were incubated with anti-Kv1.5 (rabbit polyclonal, 1:500), anti-p-Akt (rabbit monoclonal, 1:1000, anti-Akt (goat polyclonal, 1:2000), , anti-β-actin (mouse monoclonal, 1:1000) , anti-Bcl-2 (mouse monoclonal, 1:1000) antibodies over night at 4℃. Membranes were then washed with PBST three times for 10 min. After washing, the primary antibody was detected by adding an HRP-conjugated goat anti-rabbit, anti-mouse or anti-goat specific secondary antibody (1:6000) for 1.5 h. The membranes were then wash three times with PBST for 10 min and developed using the ECL™ Western blotting chemiluminescent reagent kit (Amersham Biosciences) as per manufacturer's instructions. Western blotting band intensity was determined and quantified using ImageJ software (NIH, Bethesda, MD). The region of interest was marked and measured in every lane, and the background was subtracted to give the final band intensity. Statistical comparisons were made using a two-tailed Student's t test. All data are represented as mean ± S.E.where "n" indicates the number of experiments.

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