Rottlerin protected dopaminergic cell line from cytotoxicity of 6-hydroxydopamine by inhibiting PKC δ phosphorylation

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Abstract: Objective The present study aims to investigate the role of protein kinase C δ subtype (PKC δ) phosphorylation in the process of 6-hydroxydopamine (6-OHDA)-induced dopaminergic cell death, and demonstrate the molecular basis of neurological disorders, such as Parkinson's disease. **Methods** The pheochromocytoma (PC12) cell line was employed in the present study. Cells were treated with 2 μ mol/L PKC δ inhibitor Rottlerin, 10 nmol/L protein kinase C α subtype (PKC α) inhibitor bisindolylmaleimide I, or 5 nmol/L Gö6976 that could specifically inhibit the calcium-dependent PKC isoforms, respectively. PKC δ activator phorbol-12-myristate-13-acetate (PMA, 100 nmol/L) was also used in this study. All these agents were added to the medium before cells were incubated with 6-OHDA. Cells with no treatment served as control. The cytotoxicity of 6-OHDA was determined by methyl thiazolyl tetrazolium (MTT) reduction assay and PKC δ phosphorylation levels in various groups were measured by western blotting. **Results** Bisindolylmaleimide I and Gö6976 exerted no significant attenuation on the cytotoxicity of 6-OHDA, nor any effects on PKC δ phosphorylation in PC12 cells. However, Rottlerin could inhibit the phosphorylation of PKC δ and attenuate 6-OHDA-induced cell death, and the cell viability was raised to $69.6 \pm 2.63\%$ of that in control group ($P < 0.05$). In contrast, PMA induced a significant increase in PKC δ phosphorylation and also strengthened the cytotoxic effects of 6-OHDA. The cell viability of PMA-treated PC12 cells decreased to $49.8 \pm 5.06\%$ of that in control group ($P < 0.001$). **Conclusion** Rottlerin can protect PC12 cells from cytotoxicity of 6-OHDA probably by inhibiting PKC δ phosphorylation. The results suggest that PKC δ may be a key regulator of neuron loss in Parkinson's disease.

Keywords: oxidopamine; protein kinase C δ ; Rottlerin; Parkinson's disease

1 Introduction

Parkinson's disease is a common neurodegenerative disease that has affected more and more elderly people throughout the world since the 1870s. Although it has been clear that

the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) is a pathologic hallmark of Parkinson's disease, it still remains unclear on the triggering of neurodegeneration and the precise cellular mechanisms involved in the cell loss process. Recent studies have shown that protein kinase C δ (PKC δ), a novel isoform of PKC, can be activated by dopaminergic neurotoxins such as dieldrin, 1-methyl-4-phenylpyridinium (MPP⁺) and methylcyclopentadienyl manganese tricarbonyl (MMT)^[1-4], suggesting that PKC δ activation may be related to neuron loss in Parkinson's disease.

6-Hydroxydopamine (6-OHDA) is a commonly used dopaminergic neurotoxin in animal models that induces Parkinsonism with parallel neuropathological and biochemical alterations^[5]. Therefore, an insight into the molecular mecha-

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nisms underlying 6-OHDA-induced cell death may help elucidate the pathogenesis of Parkinson's disease. In the present study, the rat pheochromocytoma cell line^[6]—PC12 cells were used to investigate the role of PKC δ in the pathogenesis of Parkinson's disease by evaluating 6-OHDA-induced threonine phosphorylation of PKC δ . The results shed light on discovering the role of PKC δ activation in the development of Parkinson's disease.

2 Materials and methods

2.1 Materials PC12 cell line which is derived from a transplantable rat pheochromocytoma, was a generous gift from Master Nye JW. Fetal bovine serum (FBS) was purchased from Biosource Inc. (USA). 6-OHDA, phosphatase inhibitor cocktail 2, Rottlerin (PKC δ inhibitor), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) and phenylmethyl-sulphonyl fluoride (PMSF) were all obtained from Sigma-Aldrich Co. (Poole, Dorset, UK). Bisindolylmaleimide I (Bis), Gö6976 and the PKC activator phorbol-12-myristate-13-acetate (PMA) were all from Merck Biosciences (Darmstadt, Germany). Antibodies against phospho-PKC δ (at the site of Thr505) and phospho-PKC (pan) (β II Ser660), the latter of which detects endogenous levels of PKC α , β I, β II, δ , ϵ , η and θ isoforms only when phosphorylated at a carboxy-terminal residue homologous to serine 660 of PKC β II, were both purchased from Cell Signaling Tech Inc. (Boston, USA). The enhanced chemiluminescence kit including horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG was obtained from Amersham Bioscience Inc. (Buckinghamshire, UK). Other chemicals were all of analytical grade and from standard commercial sources.

2.2 Cell culture and treatment PC12 cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Hyclone, Logan, USA) supplemented with 10% FBS and 1% mixture of penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. Upon 80% confluence, the cells were planted on 6-well (4 $\times$ 10⁵ cells/well) or 96-well plates (2 $\times$ 10⁴ cells/well) from the flasks. After attaching to the plate (24 h), cells were preincubated with PKC activator (PMA) or PKC inhibitors (Rottlerin 2 μ mol/L, Bis 10 nmol/L or Gö6976 5 nmol/L) for 1 h, followed by treatment with 200 μ mol/L 6-OHDA for 18 h.

Cells with no treatment served as control.

2.3 Determination of cell viability Cell viability was determined by MTT reduction assay, which is based on the conversion of MTT to blue formazan crystals by viable cells. Briefly, after the 6-OHDA treatment, cells were incubated with 0.5 mg/mL MTT prepared in fresh medium without FBS at 37 °C for 4 h, then dimethyl sulphoxide (100 μ L) was added to each well. After 10-minute agitation to dissolve the formazan, the optical density of formazan was measured at 490 nm with a Bio-Rad (US) Model 680 microplate reader. Cell viability was indicated as the percentage of OD value to that of control cells (with no treatment of 6-OHDA).

Cell viability (%) = OD (experimental group)/OD (control group) $\times$ 100%.

2.4 Cell lysate preparation and western blotting Cells in 6-well culture dishes were removed by cell scrapers. Pellet was washed with ice-cold PBS (pH 7.4) and lysed in ice-cold whole cell lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μ g/mL PMSF, 33 μ g/mL aprotinin and 1 mmol/L sodium orthovanadate, pH 7.4) supplemented with phosphatase inhibitor cocktail-2 at 4 °C for 30 min. Cell lysates were centrifuged at 12 000 g for 10 min at 4 °C. Supernatants were collected and protein concentrations were determined by DC protein assay. A total amount of 25 μ g protein was denatured by boiling and dissolved by SDS-PAGE 200 μ mol/L. After electrophoresis, the protein was transferred to a nitrocellulose membrane and the membrane was blocked in PBS (pH 7.4) containing 5% nonfat milk at 4 °C overnight. Then, the membranes were incubated with rabbit anti-phosphorylated PKC δ Thr505 (1:1 000) or mouse anti-GAPDH antibody (1:3 000) for 1 h in PBS containing 0.2% Tween-20 and 1% nonfat milk powder (blotto). Membranes were then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (1:2 000) or anti-mouse IgG (1:5 000) in blotto. Proteins were visualized by enhanced chemiluminescence technique. The intensities of bands were quantified by densitometry using Scion Image software.

2.5 Statistical analysis Data from at least 3 independent experiments were presented as mean $\pm$ SEM and analyzed by ANOVA with Post Hoc Tukey's Test using the SPSS 11.0 package (Chicago, USA). $P < 0.05$ (one-way) was regarded

as significantly different. GAPDH (36 kDa) was used to confirm the equal protein loading in each lane. Immunoband intensity was quantified by densitometry scanning using ImageJ software, and the level of PKC δ phosphorylation in the control cells was used for normalization. Densitometry data of PKC δ in different groups were expressed as the percentage to that of control samples.

3 Results

3.1 Cytotoxicity of 6-OHDA on PC12 cells As revealed by MTT reduction assay, 6-OHDA induced the decline of PC12 cell viability in a dose-dependent manner (Fig. 1A). The 50% lethal dose of 6-OHDA was about 196 $\mu\text{mol/L}$, thus the dose of 200 $\mu\text{mol/L}$ 6-OHDA was used in all the experiments.

As observed under inverted microscopy, the normal PC12 cells were polygon-like in shape and closely distributed (Fig. 1B-a). After being treated with 200 $\mu\text{mol/L}$

6-OHDA for 18 h, most PC12 cells lost their normal polygon-like shapes and exhibited round or irregular morphology. The distribution intensity also decreased (Fig. 1B-b). Moreover, the treatment of 400 $\mu\text{mol/L}$ 6-OHDA for 18 h caused the detachment and crack of PC12 cells (Fig. 1B-c).

3.2 Effect of PKC activator on cell viability and level of PKC δ phosphorylation in PC12 cells The PKC activator PMA was used to examine if the activation of PKC δ contributes to 6-OHDA-mediated cell death in PC12 cells. Results showed that similar to 6-hydroxydopamine, PMA caused the decrease of PC12 cell viability to $49.8 \pm 5.06\%$ of that in control cells ($P < 0.001$; Fig. 2A). Furthermore, when co-incubated with PMA and 6-OHDA, the PC12 cell viability decreased to $32.5 \pm 4.21\%$ of that in cells incubated with 6-OHDA alone ($P = 0.006$; Fig. 2A).

Western blotting revealed that the treatment of 6-OHDA induced the Thr505 phosphorylation of PKC δ in PC12 cells

Fig.1 Cytotoxicity of 6-OHDA on PC12 cells. A: 6-OHDA-induced cell death in PC12 cells was quantified by MTT reduction assay. 6-OHDA induced the decline of PC12 cell viability in a dose-dependent manner. Data from 3 independent experiments were presented as mean $\pm$ SEM, each being repeated 4 times. B: The morphological changes of PC12 cells in different concentrations of 6-OHDA were detected under inverted microscopy. Scale bar: 100 μm .

and this phosphorylation was strengthened by co-incubation with PMA, as shown in Fig. 2B.

3.3 Effects of PKC inhibitors on cell viability and PKC δ phosphorylation level in PC12 cells As observed under inverted microscopy, when preincubated with 2 $\mu\text{mol/L}$ Rottlerin for 1 h, followed by 200 $\mu\text{mol/L}$ 6-OHDA treatment for 18 h, part of the PC12 cells exhibited normal polygon-like morphology and were closely ranked (Fig. 3A-a). However, preincubation with 10 nmol/L bisindolylmaleimide I or 5 nmol/L Gö6976 exerted no attenuation effect on cell morphology, and most PC12 cells showed irregular characteristics, such as lower intensity, loss of normal polygon-like morphology and round or irregular shapes (Fig 3A-b, c).

As revealed by MTT reduction assay, the PKC δ inhibitor Rottlerin could significantly inhibit 6-OHDA-induced cell death, and the cell viability was increased to $69.6 \pm 2.63\%$ compared with control ($P=0.015$; Fig. 3B) at the dose of 2 $\mu\text{mol/L}$. When applied alone at the dose of 2 $\mu\text{mol/L}$, Rottlerin exerted no effect on cell viability (Fig. 3B), although the specificity of Rottlerin for PKC δ still needs to be verified and Rottlerin itself might possess toxicity^[7]. The effects of the classical selec-

tive PKC (α , βI , βII , γ) inhibitor^[8] Gö6976 (5 nmol/L) and the PKC α inhibitor bisindolylmaleimide I (10 nmol/L) on 6-OHDA-induced cell death were also investigated. As shown in Fig. 3B, neither Gö6976 nor bisindolylmaleimide I exerted any effect on the cell death induced by 6-OHDA. Besides, 6-OHDA-induced phosphorylation at the site of Thr505 in PKC δ was suppressed by Rottlerin, but not by Gö6976 or bisindolylmaleimide I (Fig. 3C).

The inhibition of PKC δ phosphorylation together with the significant increase in PC12 cell viability induced by co-incubation with Rottlerin and 6-OHDA was in concordance with the above results by using the activator of PKC δ . These findings suggest that phosphorylation at the site of Thr505 in PKC δ may be involved in neurotoxicity pathway induced by 6-OHDA in PC12 cells.

3.4 Effects of PKC inhibitors on cell viability and levels of phosphorylated PKC δ in PC12 cells treated with PMA The PKC δ inhibitor Rottlerin (2 $\mu\text{mol/L}$) significantly reduced PMA-induced cell death by 18.2% ($P < 0.001$; Fig. 4A). Meanwhile, PMA-induced PKC δ phosphorylation at the site of Thr505 was suppressed by Rottlerin.

Fig. 2 6-OHDA-induced toxicity was strengthened by PKC δ activation by PMA. **A:** Effect of PKC activator on PC12 cell viability was determined by MTT reduction assay. Data were analyzed by one-way ANOVA and post-hoc Tukey's test. $*P < 0.01$. **B:** Western blotting results of PKC δ phosphorylation induced by PKC activator. $*P < 0.01$ vs media, $^{*}P < 0.01$ vs 6-OHDA (200 $\mu\text{mol/L}$) alone.

Fig. 3 6-OHDA-induced PKC δ phosphorylation was inhibited by pre-treatment of Rottlerin. **A:** The morphological changes of PC12 cells in different conditions detected under inverted microscopy. Scale bar: 100 μm . **B:** The PKC δ inhibitor Rottlerin could significantly inhibit 6-OHDA-induced cell death, while neither Gö6976 nor bisindolylmaleimide I exerted any effect on the cell death. * $P < 0.01$. **C:** Western blotting analysis of PKC δ phosphorylation in different groups. 6-OHDA-induced phosphorylation at the site of Thr505 in PKC δ was suppressed by Rottlerin, but not by Gö6976 or bisindolylmaleimide I. * $P < 0.01$ vs media alone, # $P < 0.01$ vs 6-OHDA (200 $\mu\text{mol/L}$) alone.

Fig. 4 PMA-induced increase of PKC δ phosphorylation could be inhibited by Rottlerin, an inhibitor of PKC δ . **A:** Cell viability was evaluated by MTT reduction assay, and Rottlerin (2 μ mol/L) could significantly reduce PMA-induced cell death. * $P < 0.01$ vs media, # $P < 0.05$ vs PMA (100 nmol/L) alone. **B:** The effect of Rottlerin on PMA-induced PKC δ phosphorylation was determined by western blotting. PMA induced PKC δ phosphorylation at the site of Thr505 was specifically suppressed by Rottlerin. * $P < 0.01$ vs media alone, # $P < 0.01$ vs PMA (100 nmol/L) alone.

The inhibition of PKC δ phosphorylation and the significant increase in PC12 cell viability after co-incubation with Rottlerin and PMA confirm that phosphorylation of PKC δ at the site of Thr505 is involved in the neurotoxicity pathway induced by both 6-OHDA and PMA in PC12 cells (Fig. 4B).

4 Discussion

Parkinson's disease, also termed the shaking palsy, occurs primarily in the elderly and is characterized with severe motor disturbances and lower life quality in patients^[9,10]. With the aging of world population, the incidence of Parkinson's

disease is obviously increasing, while its pathogenesis is still unclear. As a hydroxylated derivative, 6-OHDA shares some structural similarities with dopamine and triggers the production of ubiquinone or oxygen-derived free radicals through auto-oxidation, such as hydrogen peroxide (H₂O₂), and mediates cellular oxidative stress^[11]. Due to its high affinities for several catecholaminergic plasma membrane transporters, such as the dopamine and norepinephrine transporters (NET)^[11,12], 6-OHDA can enter both dopaminergic and noradrenergic neurons and impair the catecholaminergic pathways in both the peripheral and the central nervous systems. In the dopaminergic neurotransmitter system, since it could effectively induce dopaminergic neuron death^[13], 6-OHDA is extensively used for both *in vitro* and *in vivo* investigations of neural degeneration in models of Parkinson's disease^[5,12,13]. The present study reveals that 6-OHDA could induce a dose-dependent increase in the mortality rate in PC12 cells, which is consistent with previous reports^[14-16]. The dose of 200 μ mol/L 6-OHDA was selected for further study of PC12 cell death mechanisms owing to its high capability of causing cell injury with only about 50% cell mortality rate.

PKC δ is a novel isoform of PKC^[17] that is highly expressed in dopaminergic brain regions, especially in the SNpc and corpus striatum^[18]. Moreover, the expression level of PKC δ in the central nervous system tends to increase with aging^[19]. These findings indicate that PKC δ may be potentially correlated with neuronal degeneration in Parkinson's disease. Besides, some reports have suggested that the activation of PKC δ mediates the cytotoxic effects of the oxidative stress responses^[18,20,21] induced by ultraviolet radiation^[22] or environmental neurotoxins (such as MPP⁺ and MMT) in dopaminergic neurons^[1-4,23,24].

Demonstration of the molecular mechanisms underlying the neuronal death induced by 6-OHDA, which is an endogenous dopaminergic neurotoxin, may contribute to understanding how the neurodegeneration in Parkinson's disease begins and progresses. However, little has been reported on the relationship between 6-OHDA and PKC δ phosphorylation. As revealed by the present study, although pretreatment with G6976 or bisindolylmaleimide I exerts no significant

attenuation in 6-OHDA-induced cell death, pretreatment with Rottlerin could significantly inhibit 6-OHDA-induced cell death. Furthermore, treatment of PKC activator PMA alone could decrease PC12 cell viability and aggravate 6-OHDA-induced cell death, which is similar to the response in keratinocytes and LNCaP prostate cancer cells, as described in previous studies^[25,26]. All these results are consistent with our previous findings in human neuroblastoma SH-SY5Y cells^[16], indicating that PKC δ , but not PKC α or the classic-type PKC (α , β_1 , β_2 , γ), may play a crucial role in 6-OHDA-induced PC12 cell death.

There are 3 modes of PKC activation: translocation, phosphorylation and proteolysis^[27]. However, controversy still exists concerning the most important mode of PKC activation^[3,4,16,18,19,28-30]. Recent research has suggested that PKC δ phosphorylation at the site of Tyr311 could regulate the proteolytic activation and proapoptotic function of PKC δ in dopaminergic neuronal cells^[28]. However, some researchers have suggested that phosphorylation at the site of Thr505 in PKC δ might just lead to the allosteric effect, which is one of the prerequisites for PKC δ activation^[19], and the proteolysis of PKC δ by caspase-3 represents the terminal pattern of PKC δ activation. The accumulation of PKC δ catalytic fragment is an important event in cell apoptosis^[18], particularly in H₂O₂- or MPP⁺-induced cytotoxic responses^[29,30]. Our studies demonstrate that 6-OHDA could induce PKC δ phosphorylation at the site of Thr505 in PC12 cells (Fig. 2B), which could then be inhibited by pre-treatment of Rottlerin, but not by pre-treatment of Gö6976 or bisindolylmaleimide I (Fig. 3C). On the other hand, PMA could activate PKC δ and strengthen the 6-OHDA-induced increase in PKC δ phosphorylation (Fig. 2B). The phosphorylation level of PKC δ at the site of Thr505 was inversely correlated with the viability of PC12 cells. In other words, PKC δ phosphorylation is a key downstream mediator of the toxicity of 6-OHDA in dopaminergic cells, although some other cell signal transduction kinases or other styles of PKC δ activation may also be involved. And Rottlerin could partly protect dopaminergic cell line from cytotoxicity of 6-OHDA, by inhibiting PKC δ phosphorylation.

In conclusion, our studies demonstrate that inhibition of PKC δ isoform phosphorylation by Rottlerin can offer pro-

tection from dopaminergic cell damage. Our results indicate that PKC δ may act as a novel therapeutic target for developing neuroprotective agents, and the specific and systemically active inhibitors for PKC δ isoform may serve as effective neuroprotective agents in treatment of PD.

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Rottlerin 通过抑制 PKC δ 磷酸化减轻 6 羟基多巴胺对多巴胺能细胞的毒性作用

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摘要: **目的** 观察蛋白激酶C δ 亚型(PKC δ)磷酸化在 6 羟基多巴胺(6-OHDA)引起的多巴胺能神经细胞死亡过程中的作用, 探讨帕金森病中神经元缺失的分子机制。**方法** 体外培养大鼠嗜铬细胞瘤细胞系 PC12 细胞, 观察预先加入的 PKC 抑制剂(bisindolylmaleimide I, Gö6976 及 Rottlerin)和激动剂佛波酯对 6-OHDA 毒性作用的影响, 噻唑蓝比色法检测细胞存活率, 免疫印迹法观察磷酸化 PKC δ 的表达。**结果** PKC δ 抑制剂 Rottlerin (2 μ mol/L)可抑制 PKC δ 的磷酸化, 减轻 6-OHDA 引起的细胞死亡, 细胞存活率上升至 $69.6 \pm 2.63\%$ ($P < 0.05$)。PKC α 抑制剂 bisindolylmaleimide I 和钙依赖性 PKC 抑制剂 Gö6976 对 6-OHDA 的毒性作用及 PKC δ 磷酸化均无显著影响, 而 PKC δ 激活剂佛波酯(100 nmol/L)能提高 PKC δ 磷酸化水平, 加重 6-OHDA 的损害作用, 使细胞存活率下降至单用 6-OHDA 组水平的 $49.8 \pm 5.06\%$ ($P < 0.001$)。**结论** Rottlerin 能抑制 PKC δ 的磷酸化, 进而减轻 6-OHDA 对多巴胺能神经细胞死亡的诱导作用, 说明 PKC δ 505 位点丝氨酸的磷酸化是 6-OHDA 发挥毒性作用的关键, 提示 PKC δ 在帕金森病病人神经元缺失中起重要作用。

关键词: 羟多巴胺; 蛋白激酶 C, δ 亚型; Rottlerin; 帕金森病