

Neuroprotective effects of porphyran derivatives against 6-hydroxydopamine-induced cytotoxicity is independent on mitochondria restoration

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Abstract: We previously reported that acetylated and phosphorylated derivatives of porphyran extracted from *Porphyra haitanensis* exhibit antioxidant activity in cell-free system. The aim of the present study was to investigate the neuroprotective effects of porphyran and its derivatives on 6-hydroxydopamine (6-OHDA)-induced cytotoxicity. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell viability. Changes in the mitochondrial transmembrane potential ($\Delta\Psi_m$) were measured by rhodamine123 using flow cytometry. The results showed that porphyran and its two derivatives, acetylated porphyran (AP) and phosphorylated porphyran (PP) (<1 mg/mL) alone did not have any toxic effects on MES23.5 cells. The cell viability decreased when cells were treated with 25 $\mu\text{mol/L}$ 6-OHDA. Both AP and PP, rather than porphyran, significantly antagonized 25 $\mu\text{mol/L}$ 6-OHDA-induced cytotoxicity. However, neither AP nor PP could antagonize 6-OHDA-induced mitochondrial transmembrane potential ($\Delta\Psi_m$) collapse. None of the three materials were effective on cell survival when cells were cotreated with 75 $\mu\text{mol/L}$ 6-OHDA. These results suggest that two derivatives of porphyran, AP and PP, could antagonize the weak toxicity of 6-OHDA on MES23.5 dopaminergic cells, possessing minor neuroprotective effects independent of mitochondria restoration.

Keywords: Porphyran; acetylated porphyran (AP); phosphorylated porphyran (PP); 6-hydroxydopamine (6-OHDA); neuroprotective effects

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disease characterized by the progressive degeneration of the nigral dopaminergic neurons, leading to dopamine depletion in the striatum and various clinical symptoms. PD is associated with several risk factors including aging, genetic susceptibility or environmental factors, which could act synergistically in promoting degeneration of dopaminergic neurons (1-5). Among them, oxidative stress and free radicals are one of the most common mechanisms in the pathogenesis of PD (6,7). Therefore, natural components that exhibit

antioxidant properties are drawing great attention as potential candidates for the treatment of PD.

Polysaccharides which are the main components of algae have been demonstrated to serve as free-radical scavengers and antioxidants against oxidative damage in living organisms (8,9). Some modifications, such as acetylation and phosphorylation, could enhance its biological functions (10,11). *Porphyra haitanensis* is an important economic alga in southern China. We previously reported that acetylated and phosphorylated derivatives of porphyran [acetylated porphyran (AP) and phosphorylated

porphyrin (PP)] extracted from *Porphyra haitanensis* exhibit antioxidant activity. The data suggested that AP showed more significant effect on superoxide radical and reducing power than porphyrin, while phosphorylated derivatives had much stronger hydroxyl radical scavenging activities than porphyrin in cell-free system (12). In the present study, we aim to study whether porphyrin and its two derivatives could exert neuroprotective effects on dopaminergic cells against 6-OHDA-induced neurotoxicity, which are commonly used in inducing PD models. MES23.5 cells were used as the experimental neuronal model, a dopaminergic cell line hybridized from murine neuroblastoma-glioma N18TG2 cells with rat mesencephalic neurons exhibiting several properties similar to the primary neurons originated in the substantia nigra (13). The data presented here using 6-OHDA-treated MES23.5 cells as a model for PD suggest that two derivatives of porphyrin, AP and PP, could antagonize the weak toxicity of 6-OHDA on MES23.5 dopaminergic cells, possessing minor neuroprotective effects independent of mitochondria restoration.

Materials and methods

Materials

Porphyrin, AP and PP were prepared as described previously (14). Dulbecco's modified Eagle's medium Nutrient Mixture-F12 (Ham; DMEM/F12) was from Gibco (Gibco, Grand Island, NY, USA). MES23.5 cells was a dopaminergic cell line hybridized from murine neuroblastoma-glioma N18TG2 cells with rat mesencephalic neurons (13). It was kindly provided by Dr. Weidong Le (Department of Neurology and Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas, USA). All other chemicals and reagents were of Sigma Chemical Co (St Louis, Missouri, USA) or the highest grade available from local commercial sources.

Cell culture

MES23.5 cells were cultured in DMEM/F12 containing Sato's components growth medium supplemented with 5% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C, in a humid 5% CO₂, 95% air environment. For experiments, cells were seeded at a density of 1×10⁵/cm² in the plastic flasks. Porphyrin, AP and PP were dissolved in serum-free DMEM/F12 to a final concentration of 10 mg/mL, stored at -20 °C. Then they

were diluted with DMEM/F12 without serum to different concentrations (1, 10⁻¹, 10⁻² mg/mL) before usage. To study the protective effects of porphyrin, AP and PP, they were added to the medium with 6-OHDA (25 or 75 µmol/L) for 24 hours, respectively.

Cell viability assay

Cell viability was determined by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MES23.5 cells were seeded in a 96-well plate at a density of 1×10⁴ cells/well. After attachment, cells were treated with different concentrations of porphyrin, AP, PP alone, or together with 25/75 µmol/L 6-OHDA in DMEM/F12 without serum supplement for 24 hours, respectively. After incubation, cells were incubated in MTT (5 mg/mL) for 4 hours at 37 °C, the medium was removed and 100 µL of DMSO was added to each well and the formazan dye crystals were solubilized. The absorbance was measured at 570 nm by colorimetric assay (Spectra Max M5, Molecular Devices).

Mitochondrial transmembrane potential ($\Delta\Psi_m$) measurement

Changes in the $\Delta\Psi_m$ with various treatment in MES23.5 cells were measured by rhodamine123 using flow cytometry (Becton Dickinson, USA) as described before (15). The uptake of rhodamine123 into mitochondria is an indicator of the $\Delta\Psi_m$. Fluorescent intensity was recorded at 488 nm excitation and 525 nm emission wavelength [fluorescence (FL1)]. The gated region M1 was set automatically as a threshold when cells without rhodamine123 incubation were analyzed. That means most of the cells without rhodamine123 fluorescence should be located in M1 region and most of the normal cells with rhodamine123 fluorescence should be located in M2 region. Cells treated as described above were incubated in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (HBS) containing rhodamine123 in a final concentration of 5 µmol/L for 30 minutes at 37 °C and followed by washing twice with HBS. For analysis, data was collected as the percentage of cells which were rhodamine123 fluorescence-positive using CellQuest Software.

Statistical analysis

Data were presented as means ± SEM. Normal distribution

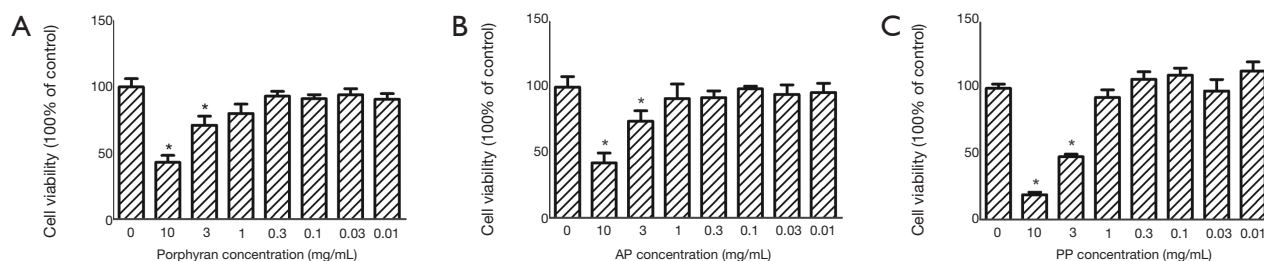


Figure 1 MTT analysis of cell viability in porphyran, AP and PP treated MES23.5 cells. The cell viability was reduced in 10 and 3 mg/mL porphyran (A), AP (B) or PP (C) treated groups. However, there was no difference in the other five concentrations (1, 0.3, 0.1, 0.03, 0.01 mg/mL). Data were presented as mean \pm SEM of six independent experiments. *, $P < 0.01$, compared with the control. Abbreviations: AP, acetylated porphyran; PP, phosphorylated porphyran.

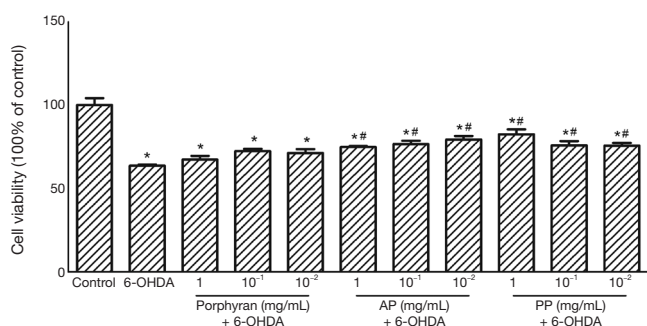


Figure 2 Changes in cell viability of MES23.5 cells with 25 μ mol/L 6-OHDA and different concentrations of porphyran, AP and PP cotreatment. The cell viability was reduced 36.2% \pm 0.6% with 25 μ mol/L 6-OHDA treatment. An increase in cell viability was observed when cells were cotreated with either AP or PP (1, 10^{-1} , 10^{-2} mg/mL). Porphyran itself did not have any effect. Data were presented as mean \pm SEM of six independent experiments. *, $P < 0.05$, compared with the control; #, $P < 0.05$, compared with 6-OHDA group. Abbreviations: AP, acetylated porphyran; PP, phosphorylated porphyran.

of data and homogeneity of variance were verified by normality test and homogeneity test ($P > 0.05$). One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the differences between different groups. A probability value of $P < 0.05$ was considered to be statistically significant.

Results

Effects of porphyran, AP and PP on cell viability in MES23.5 dopaminergic cells

Using MTT assay, we detected the changes in cell

viability of MES23.5 cells treated with different concentrations of porphyran, AP and PP. When treated with porphyran, AP and PP lower than 1 mg/mL respectively, cell viability was unchanged compared with that of control (Figure 1). However, a significant reduction in cell viability was observed when cells were treated with 3 mg/mL or 10 mg/mL porphyran (Figure 1A), AP (Figure 1B) and PP (Figure 1C).

AP and PP rather than porphyran antagonized 25 μ mol/L 6-OHDA-induced cytotoxicity in MES23.5 cells

The neuroprotective effects of porphyran and its derivatives against 6-OHDA-induced cytotoxicity were assessed in cultured MES23.5 cells by MTT assay. As shown in Figure 2, the cell viability was reduced to 63.7% \pm 1.8% in 25 μ mol/L 6-OHDA-treated group. When treated with 1, 10^{-1} , 10^{-2} mg/mL AP or PP, the cell viability could be observed a slightly increase compared with that of control, indicating that they possess a certain neuroprotective property. However, raw porphyran did not have any protective effect (Figure 2).

AP and PP could not prevent 6-OHDA-induced mitochondrial transmembrane potential ($\Delta\Psi_m$) collapse

$\Delta\Psi_m$ is the marker of mitochondria function, which is involved in a variety of key events in oxidative stress. Next in order to explore the mechanisms underlying the neuroprotective effects of AP and PP, we measured the changes in $\Delta\Psi_m$ of the above groups. Unfortunately, neither AP nor PP pretreatment could antagonize 6-OHDA-induced $\Delta\Psi_m$ collapse (Figure 3).

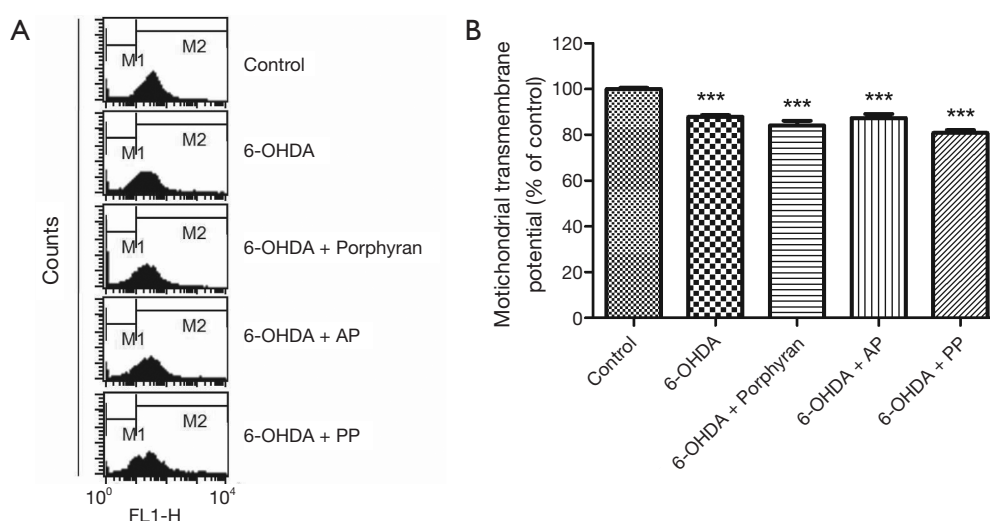


Figure 3 Mitochondrial transmembrane potential ($\Delta\Psi_m$) changes of MES23.5 cells with 25 $\mu\text{mol/L}$ 6-OHDA and 10^{-2} mg/mL porphyrin, AP and PP cotreatment. (A) Representative of the fluorometric assay on $\Delta\Psi_m$ of different groups. No changes were observed in 25 $\mu\text{mol/L}$ 6-OHDA and 10^{-1} mg/mL porphyrin, AP and PP cotreatment; (B) statistical analysis. Data were presented as mean \pm SEM of three independent experiments. Fluorescence values of the control were set to 100%. ***, $P < 0.001$, compared with the control. Abbreviations: AP, acetylated porphyrin; PP, phosphorylated porphyrin.

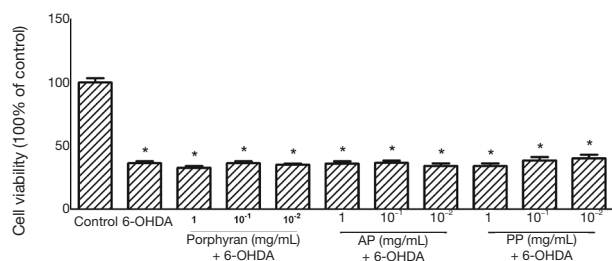


Figure 4 Changes in cell viability in MES23.5 cells with 75 $\mu\text{mol/L}$ 6-OHDA and different concentrations of porphyrin, AP and PP cotreatment. The cell viability was reduced about $63.1\% \pm 2.3\%$ treated with 75 $\mu\text{mol/L}$ 6-OHDA. No changes were observed in porphyrin, AP and PP (1, 10^{-1} , 10^{-2} mg/mL)-treated groups. Data were presented as mean \pm SEM of six independent experiments. *, $P < 0.05$, compared with the control. Abbreviations: AP, acetylated porphyrin; PP, phosphorylated porphyrin.

Porphyrin, AP and PP could not antagonize 75 $\mu\text{mol/L}$ 6-OHDA-induced cytotoxicity in MES23.5 cells

The neuroprotective effects of porphyrin and its derivatives against 6-OHDA-induced cytotoxicity were further assessed in a high dose of 6-OHDA treatment. The cell viability was dramatically reduced to 37% with 75 $\mu\text{mol/L}$ 6-OHDA treatment. Unfortunately, none of them showed

cytoprotective effects against 6-OHDA-induced reduction in cell viability (Figure 4).

Discussion

In this study we demonstrated that two derivatives of porphyrin (acetylated and phosphorylated forms) could exert neuroprotective effects against a lower dose of 6-OHDA-induced neurotoxicity. However, these effects were weak when compared with the classical antioxidant N-acetylcysteine (NAC) or other natural derivatives (such as curcumin, Rg1, myricetin *et al.*) reported before in our laboratory (15-18). We also demonstrated that both AP and PP could not antagonize 6-OHDA induced $\Delta\Psi_m$ collapse, indicating this neuroprotection was independent of mitochondria restoration.

Oxidative stress was implicated in the pathogenesis of PD (19). 6-OHDA lesioning is a well established method for preparing PD models (20,21) which is related to both intracellular or extracellular oxidation, and it was also shown that 6-OHDA could inhibit the mitochondrial respiratory chain through inhibiting Complex I, uncoupling oxidative phosphorylation and collapsing mitochondrial membrane (22). In the present study, 6-OHDA was chosen as a neurotoxin to induce oxidative damage in MES23.5 dopaminergic cells. We previously reported porphyrin had

strong antioxidant activity (23). However, it may not be a simple stoichiometric reaction and the function of porphyrin was biphasic with higher doses causing damage as shown in *Figure 1*. In that cell-free system, both raw material and certain derivatives exerted scavenging effects for superoxide/hydroxyl radical and reducing power, with derivatives being more powerful. In accordance with these results, we revealed in the present study that both AP and PP, but not porphyrin itself, effectively antagonized 25 $\mu\text{mol/L}$ 6-OHDA-induced cytotoxicity. To explore the underlying mechanisms, we further measured the mitochondrial transmembrane potential in 25 $\mu\text{mol/L}$ 6-OHDA-treated cells and 10^{-2} mg/mL AP or PP co-treated cells. However, both AP and PP could not prevent 6-OHDA-induced $\Delta\Psi\text{m}$ collapse, indicating that the protective mechanism of AP and PP might be due to the antioxidative effects as what they showed in the previous publication (12), however, irrelevant to mitochondria function. Moreover, the protective effect could only be achieved in a low concentration (25 $\mu\text{mol/L}$) of 6-OHDA-treated cells but not be observed in cells with dramatically decreased cell viability due to a higher concentration (75 $\mu\text{mol/L}$) of 6-OHDA treatment.

In summary, the present study demonstrates that two derivatives, AP and PP, exert minor cytoprotective effects, even if they have stronger antioxidant activities than raw material. Mitochondrial restoration was not involved in this process.

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Authors' contributions: W. Wang performed cell viability assay and flow cytometric measurement of mitochondrial transmembrane potential. N. Song performed cell viability assay and analyzed data. F. Jia performed cell viability assay. J. Xie participated in the experiment design and drafted the manuscript. Q. Zhang participated in the experiment design. Hong Jiang participated in the experiment design, analyzed data, and drafted the manuscript.

Disclosure: The authors declare no conflict of interest.

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