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Isoflavone Attenuates the Nuclear Transcription Factor Kappa B (NF-κB) Activation on MPP+-Induced Apoptosis of PC12 Cells

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Abstract

Objective: To explore the underlying molecular mechanisms of cellular response to the challenge by 1-methyl-4-phenylpyridinium (MPP^+)-induced apoptosis of PC12 cells, an *in vitro* cell model for Parkinson's disease, and the effect of NF-κB activation on the protection of Parkinson's disease by Isoflavone (I). **Methods:** PC12 cells were used to establish the cell model of Parkinson's disease, and are divided into five groups: control group; MPP^+ group; I (Isoflavone) + MPP^+ group; I group; SN-50 + MPP^+ group. The content of NF-κB in PC12 cells was determined by immunocytochemistry; The viability of PC12 cells after treated with cell-permeable NF-κB inhibitor SN-50 and cell viability were measured by MTT assay; the expression levels of NF-κB p65 in cytoplasm and nuclear fractions were evaluated by western blot analysis; the mRNA expression of NF-κB p65 was analyzed by in situ hybridization (ISH). **Results:** Compared with the control group, the protein of NF-κB p65 both in cytoplasm and in nuclei was significantly higher than in I + MPP^+ and MPP^+ groups; similarly, the mRNA expression level of NF-κB p65 gene was also significantly higher; moreover, the protein expression of NF-κB p65 was much lower in I group ($P < 0.05$). In addition, compared with the MPP^+ group, the protein of NF-κB p65 was significantly lower in I + MPP^+ group, the mRNA expression level of NF-κB p65 gene was also significantly lower, and the protein expression level of NF-κB p65 was much lower in I + MPP^+ group ($P < 0.05$); however, there was no significant difference between control group and I + MPP^+ group ($P > 0.05$). **Conclusion:** NF-Κb

*The authors equally contributed to the work.

activation is essential to MPP^+-induced apoptosis in PC12 cells; but Isoflavone can inhibit the cell damage to some extent to execute its protective function, which may be involved in nigral neurodegeneration in patients with Parkinson's disease.

Keywords

Isoflavone, PC12 Cell, MPP^+, Apoptosis, NF-*κ*B p65, Nuclear Transcription Factor Kappa B, Parkinson's Disease

1. Introduction

The pathological mechanisms of Parkinson's disease (PD) remain unclear in recent years. Although recent studies have documented that the degeneration of neurons in the substantia nigra is highly correlated with denaturation and apoptosis of neurons [1]. In 1997, Hunot [2], a US scholar, have found that the immune response of NF-*κ*B in dopaminergic nuclei of PD patients is enhanced by 70 times when compared with that in the control group ,so that NF-*κ*B involved in the pathogenesis of PD has been first put forward. Although the important role of NF-*κ*B in degenerative diseases remains unclear, it has gained extensive attention from PD researchers.

In our previous study, we use 1-methyl-4-phenylpyridinium (MPP^+)-induced apoptosis of PC12 cells as the model of Pakinson's disease. Isoflavone is one of the ingredients from soybean. Studies [1] [2] [3] have showed that Isoflavone has some effect of estrogen and have many potential clinical implications with mechanism of action, especially in the treatment and prevention of diabetes, cardiovascular diseases, cancer, osteoporosis, neuroprotection. Our previous study has indicated that estrogen had the protective effect to cell model of Parkinson's disease, but estrogen perhaps brings some side effects and restrict its use on clinical, so many researchers are looking for better substitute for estrogen, which not only has the effect of estrogen, but also has no side effect of it. Fortunately researchers found plant-estrogen [1], Isoflavone is one of the plant estrogen which has the same effect.

In the present study, whether the involvement of NF-*κ*B p65 in the toxicity of MPP^+ exposure to PC12 cells and the effect of phytoestrogen Isoflavones on this process was systematically explored.

2. Materials and Methods

2.1. Cell Culture

Both naive and differentiated PC12 cells were cultured at 37˚C in RPMI 1640 media (Sigma USA) supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% carbon dioxide in room air. The dispersed cells were seeded onto colla-

gen-coated 96-well plates at a density of 3×10^4 cells/well, and cultured under various drug regimen combinations. The cultured cells were divided into five groups including control (vehicle), MPP^+ (250 mmol/L), Isoflavone (Guangzhou Aipu technology company) combined with MPP^+ (10 mM Isoflavone and 250 mmol/L MPP^+), and Isoflavone alone groups (10 mM), SN-50 + MPP^+ (Sigma USA) group (10 mM SN-50 and 250 mmol/L MPP^+).

2.2. Establishment of Cell Model with Parkinson's Disease

Based on the classical model establishment strategy of MPP^+-induced cell damage [1], the cells were treated with 250 μM MPP^+ (Sigma USA) to induce the apoptosis of cell model with PD for the future experimental use, which is accordant with the international general model standard.

2.3. Thiazolyl Blue Tetrazolium Bromide (MTT) Assay of Cell Viability

After PC12 cells were incubated with MTT solution (5 mg/mL, Sigma USA) at 37˚C for 4 h, Treated with Formazan crystal dissolved in 100 μL dimethyl sulfoxide (DMSO). The absorbance of MTT was measured at 570 nm using a DG-3022A ELISA plate reader (Beijing QinYe Technology Company). The reduction of MTT absorbance was expressed as a percentage based on the normalization of the control as 100%. The cell viability was evaluated by OD data.

2.4. Expression of NF-*κ*B p65 in Cytoplasm and Nuclei Evaluated by Western Blot

The PC12 cell culture was conducted using previously reported methods [4] to extract the cytoplasm and nuclear fractions. Cells (10^6/sample) were harvested by mechanical scraping in PBS solution (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) at 4˚C. After centrifugation at 1500 g for 5 min, the cell pellets were re-suspended using 400 μL of chilled buffer [10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethysulforiyl fluoride (PMSF), pH 7.9. Following standing on ice for 15 min, the suspended solution was added with 0.025 mL of NP-40 (10%), shaken through vortex for 10 s, and centrifuged for 30 s to collect the supernatant as the cytoplasm fraction.

The precipitate was re-suspended with 0.05 mL of chilled buffer (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 25% glycerol, pH 7.9). The suspended solution was greatly shaken through vortex for 15 min and then centrifuged for 15 min to obtain the supernatant as the nuclear fraction. The protein was quantified using BCA kit.

After the concentrations of proteins in samples were determined, the 4× loading buffer (0.1 M Tris, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 10% *β*-ME, pH 6.8) was added to the samples with the sequential shaking for 20 s and boiling at 100˚C for 5 min. Then, the samples at the identical protein amount was loaded in each well and separated through electrophoresis at the constant voltage of 100 V for 1.5 h. If the indicator was moved to the junction point be-

tween condensed gel and separation gel, the voltage was changed to 140 V for electrophoresis for 3 h. The proteins were sequentially transferred to PVDF membrane at the constant current of 200 mA with the transferring time of 30 min. The PVDF membrane with proteins was blocked with 30% BSA-TBS at the room temperature with slight shaking for 30 min. After blocking, the PVDF membrane was incubated with NF-κB p65 primary antibody (Sigma USA) overnight and then washed with TBS buffer (50 mM Tris, 100 mM NaCl, pH 7.5) for 3 times and probed with alkaline phosphatase-labeled goat anti-mouse secondary antibody (3% BSA, 1:30,000, Sigma USA) at 37˚C for 1 h. After washing with TBS buffer for 3 times with 5 min each time, the PVDF membrane was incubated the mixture containing 4 mL of alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5), 100 μL of 30 mg/L color-developing solution (5-bromo-4-chloro-3-indolylphosphonic acid-p-aminotoluene salt, BCIP), and 100 μL of 15 mg/mL nitro blue tetrazolium chloride (NBT) at light-free environment for 5 - 10 min. TBS washing was used to terminate the reaction and the PVDF membrane was dried at cool environment. The immune response signal was detected using Western blot detection system. NF-κB p65 was quantitatively analyzed based on the bands imaged by Shimadzu dual wave color scanner.

2.5. *In Situ* Hybridization Detection of NF-κB P65 mRNA

Cells were washed with PBS three times (2 min for each time) and fixed with 4% paraformaldehyde/0.1 MPBS (containing 0.1% DEPC) for 20 - 30 min. The cells were subsequently processed with 0.5% H_2O_2/methanol at room temperature for 30 min, in order to inactivate endogenous peroxidase enzymes. The slides containing cells were added 3% pepsin diluted with citric acid for digestion at 37˚C for 15 min. The slides were washed with PBS containing 0.3% Triton X-100 for three times (5 min for each time). NF-κB p65 digoxigenin-labeled in situ hybridization solution (20 mL) was added to each slide. Hybridization was kept at 37˚C overnight. After washing with SSC, 10% normal goat serum (PBS diluted) was added to the slides, followed by the addition of mouse anti-digoxigenin at 37˚C for 60 min. After PBS washing, biotinylated goat anti-mouse IgG and SABC were added. The slides were stained with hematoxylin and visualized with DAB. Brown cytoplasm proved positive results. Under a light microscope, 10 images were randomly selected from each slide. The images were analyzed by an HPIAS-1000 high-resolution system (Beijing QinYe Technology Company) to measure the optical density of 100 - 150 cells. The mean optical density of these cells was calculated.

2.6. Statistical Analysis

Data were expressed as mean ± standard deviation (M ± SD), and statistical analysis of the data for multiple comparisons was performed by ANOVA. For single comparison, student t test was used. Categorical data were analyzed with Chi-square test. $P < 0.05$ was considered as statistically significant difference.

3. Results

1) The viability and metabolic state of cells analyzed by MTT (Table 1)

Cell viability in MPP^+ group was lower than that in the control ($P < 0.05$), I, I + MPP^+ ($P < 0.01$), SN-50 + MPP^+ groups ($P < 0.05$).

There was no significant difference in cell viability between I + MPP^+ and control groups ($P > 0.05$).

2) The expression level of NF-κB p65 protein evaluated by Western blot (Table 2; Figure 1)

The quantification of optimal density of NF-κB protein(whatever in cytoplasm or in nuclei) in MPP^+ group was higher than that in the control ($P < 0.05$), I, I + MPP^+ ($P < 0.01$), SN-50 + MPP^+ groups ($P < 0.05$).

There was no significant difference in cell viability between I + MPP^+ and control group ($P > 0.05$).

There was no significant difference between I + MPP^+ and control group ($P > 0.05$).

3) The mRNA expression of NF-κB evaluated by in situ hybridization (Figure 2; Table 3)

The content of NF-κB p65 mRNA were higher in MPP^+ group than those in the control ($P < 0.05$), I ($P < 0.01$), I + MPP^+ ($P < 0.01$), SN-50 + MPP^+ ($P < 0.05$).

There was no significant difference between I + MPP^+ and control group ($P > 0.05$).

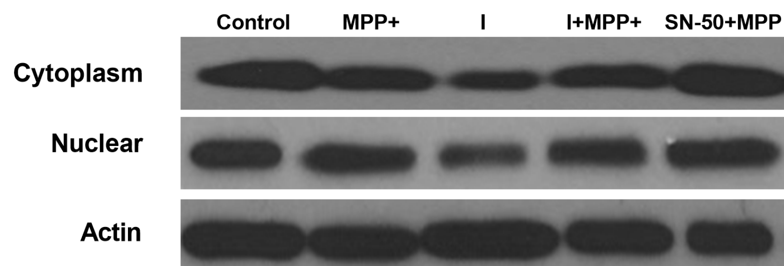

Figure 1. The protein expression levels of NF-κB p65 were higher in MPP^+ group than those in the control ($P < 0.05$), I ($P < 0.01$), I + MPP^+ ($P < 0.01$), SN-50 + MPP^+ ($P < 0.05$).

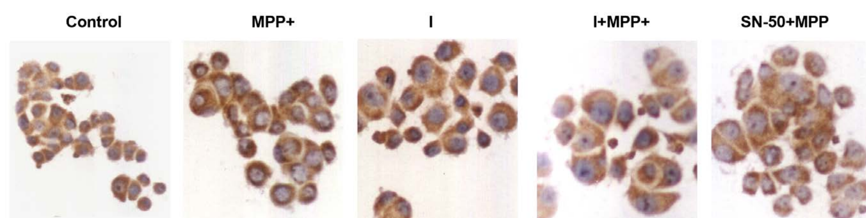

Figure 2. The NF-κB p65 positive PC12 cells were stained in brown color both in cytoplasm and nuclei (DAB × 200). The mRNA expression levels of NF-κB p65 were higher in MPP^+ group than those in the control ($P < 0.05$), I ($P < 0.01$), I + MPP^+ ($P < 0.01$), SN-50 + MPP^+ ($P < 0.05$). There was no significant difference between I + MPP^+ and control group ($P > 0.05$).

Table 1. The viability and metabolic state of cells analyzed by MTT ($\bar{x} \pm s$).

Group	Control	MPP+	I	I + MPP+	SN-50 + MPP+
OD data	0.49 ± 0.11	0.30 ± 0.07	0.61 ± 0.17	0.56 ± 0.16	0.45 ± 0.12

Table 2. The quantification of optimal density of NF-*κ*B protein ($\bar{x} \pm s$).

Group	Control	MPP+ (250 μm)	I (10 nm)	I + MPP+	SN-50 + MPP+
Cytoplasm	62 ± 12	90 ± 17	41 ± 9	69 ± 17	72 ± 15
Nuclei	57 ± 11	72 ± 15	36 ± 4	52 ± 14	69 ± 18

Table 3. The content of NF-*κ*B p65 mRNA ($\bar{x} \pm s$).

Group	Control	MPP+	I	I + MPP+	SN-50 + MPP+
Optical density	0.302 ± 0.051	0.654 ± 0.072	0.113 ± 0.032	0.332 ± 0.067	0.137 ± 0.065

4. Discussion

NF-*κ*B is one of the members of Rel transcription factor family involved in a wide range of gene regulation including immune and defense mechanisms [5]. NF-*κ*B is a ubiquitous multidirectional nuclear transcription regulator responsible for regulating gene expression of cytokines, chemokines, adhesion molecules, growth factors, immune receptors, oxidative stress-related enzymes, transcription factors, and stress-related proteins, which are involved in many pathological and physiological processes. The most extensively studied NF-*κ*B complex including p50 and p65 subunits contains a segment with 300 amino acids, and both subunits have the homology with Rel proto-oncogene product. The p50 subunit can bind to DNA, while p65 subunit can interact with I*κ*B, as the inhibitor of NF-*κ*B. In many cells, p50/p65 heterodimer can co-exist with I*κ*B in the cytoplasm in a complex form. The activation of NF-*κ*B is regulated by I*κ*B. I*κ*B is present in the cytoplasm. Its activation state can be divided into inducible and structural forms. Inducible I*κ*B in the cytoplasm can inhibit its binding with NF-*κ*B in the non-activated state; I*κ*B in the structural form can bind to DNA in nuclei after being activated although it has no I*κ*B. Activated NF-*κ*B can be transported into nuclei retrogradely by axons, thereby playing its signal transduction in the central nervous system and executing its biological role.

Many evidences suggest that [6] [7] [8] NF-*κ*B may accelerate the progression of apoptosis. Many NF-*κ*B agonists, such as tumor necrosis factor, ceramide and H_2O_2, eventually induce apoptosis. Similarly, many apoptosis-inducing genes, such as Fas/Apo-1, c-myc, p53 and Caspase, are induced by NF-*κ*B. Other studies have drawn the opposite conclusion. Some studies [9] [10] have pointed out that NF-*κ*B is a newly discovered important apoptosis antagonist, which can transcriptionally regulate the expression of a series of genes associated with cell survival, thereby blocking the signal pathways of apoptosis. Some scholars [11] have also pointed out that the activation of NF-*κ*B has high correlation with neuronal apoptosis of PD patients. Our *in vitro* and *in vivo* studies have demon-

strated that [11] [12] that estrogen can protect dopaminergic neurons from MPP^+-induced damage and apoptosis. However, during the process of executing protective roles in the present of estrogen, the roles of NF-κB as an important factor are still not fully understood.

Our results confirmed MPP^+-induced NF-κB activation through NF-κB blocker SN-50 influencing cell membrane permeability. Although cell viability had no significant difference during MPP^+ treatment, SN-50 hindered the toxicity of MPP^+ on PC12 cells to certain extents. Moreover, cell viability in the Isoflavone group was significantly higher than that in other groups, but there was no significant difference between I + MPP^+ group and SN-50 + MPP^+ group. Meanwhile, the expression levels of NF-κB p65 protein and mRNA in Isoflavone group were significantly lower than those in other groups. Similarly, there was no significant difference between I + MPP^+ group and SN-50 + MPP^+ group. All of these results suggest that MPP^+ can induce the activation of NF-κB and may accelerate cellular injury to some extents. The activated NF-κB not only can be expressed in the cytoplasm, but also can be transported retrogradely by axons into nuclei, thereby correspondingly regulating gene transcription and leading to the increase of nuclear NF-κB. As a protective factor, the protective effect of Isoflavone on neuron cell damage can be realized through blocking the activation of NF-κB to some extents; however, its underlying mechanisms are still unclear. The speculated mechanisms may be related to the follows: 1) direct inhibition of NF-κB p65 activation, 2) promoted binding between NF-κB p65 and its inhibitory protein IκB, 3) blocked transportation of NF-κB from axons to nuclei, 4) blockage of NF-κB-mediated signal transduction and immune responses. On the other hand, NF-κB may play a variety of roles in cell survival of neurons, which depends on different experimental conditions, cell types, activation patterns and kinetic properties [13] [14]. Whether NF-κB executes the protective role or the damaged role has tissue specificity, and dose, spatial or time dependence is still unclear. In spite of this, our study have shown that the protective effect of Isoflavone on MPP^+-induced damage of PC12 cells is highly correlated with the inhibitory effect of Isoflavone on the activation of NF-κB p65, which greatly stimulates us to explore the relationships among Isoflavone, NF-κB and PD.

Based on the roles of NF-κB in regulating apoptotic process in neurodegenerative diseases, NF-κB is expected to become a novel pharmacological target of PD treatment. Therefore, how to control the appropriate activation range of NF-κB, how to selectively block NF-κB nuclear translocation, and how to modulate the specific role and the spatial-time correlation of NF-κB in PD disease need to be further explored. We will do further work to solve these problems in the future.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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