Involvement of ERK1/2 and p38 MAPK in up-regulation of 14-3-3 protein induced by hydrogen peroxide preconditioning in PC12 cells

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Abstract: Objective To investigate the protective effects of hydrogen peroxide preconditioning (HPP) on the pheochromocytoma (PC12) cells treated with 1-methyl-4-phenylpyridinium (MPP⁺) and to explore the potential mechanisms. **Methods** The viability and apoptosis of PC12 cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and 4',6'-diamidino-2-phenylindole (DAPI) staining, respectively. The expressions of 14-3-3 protein and phosphorylated p38 mitogen-activated protein kinase (MAPK) were determined by Western blot. Enzyme-linked immunosorbent assay (ELISA) was used to measure the activity of extracellular signal-regulated protein kinase 1/2 (ERK1/2). **Results** The cell viability decreased and the number of apoptotic cells increased dramatically in MPP⁺ group compared with that in Control group. HPP induced a significant increase in cell viability and a marked decrease in population of apoptotic cells of the MPP⁺-treated PC12 cells, accompanied with up-regulation of 14-3-3 protein and increase of ERK1/2 and p38 MAPK activities. The 14-3-3 protein expression was positively correlated with the phosphorylation of ERK1/2. Furthermore, inhibition of the ERK1/2 with PD98059 abolished the 14-3-3 protein up-regulation in PC12 cells induced by HPP. **Conclusion** HPP protects PC12 cells against MPP⁺ toxicity by up-regulating 14-3-3 protein expression through the ERK1/2 and p38 MAPK signaling pathways.

Keywords: hydrogen peroxide preconditioning; 14-3-3 protein; ERK1/2; p38 mitogen-activated protein kinase; PC12 cell

1 Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders in the elderly population. Although its etiology remains not fully understood, oxidative stress-induced apoptosis of dopaminergic neurons has received the most attention^[1]. Oxidative stress has been known to participate in dopaminergic neuronal damage mediated by reactive oxygen species (ROS). Hydrogen peroxide (H₂O₂), which belongs to ROS, may be formed from dopamine via oxidative deamination by monoamine oxidase. However, recent reports have found that hydrogen peroxide preconditioning (HPP) protected human proximal tubular cells^[2] and rat fibroblast L-cells^[3] against lethal oxidant injury. The protection of HPP has also been demonstrated in the PC12 cells exposed to H₂O₂^[4] and dopamine^[5], but the involved mechanisms are not understood yet. HPP, maybe, similar to ischemic/anoxic preconditioning, exerts its protective effects through up-regulating some endogenous protective proteins.

14-3-3 Proteins represent a family of acidic intracellular proteins with molecular weight of 28–33 kDa. It is abundantly expressed in the brain^[6]. The 14-3-3 displays important anti-apoptotic characteristics through interaction with pro-apoptotic molecules such as BCL-xL/BCL-2-associated death promoter (BAD), forkhead transcription factor-1 (FKHRL-1), apoptosis signal-regulating kinase 1 (ASK1), and A20^[7]. Furthermore, some studies have shown that 14-3-3 protein up-regulation appeared in the organism/cell injury induced by cytokine stress^[8], ischemic/anoxic^[9], lipopolysaccharide^[10], and so on. Additionally, we previously showed that

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overexpression of 14-3-3 protein protected PC12 cells against 1-methyl-4-phenylpyridinium (MPP⁺) toxicity^[11]. These observations suggest that 14-3-3 protein may be associated with cytoprotection.

However, it is not clear whether HPP-induced cellular protection involves 14-3-3 protein and which pathways determine 14-3-3 protein expression during HPP. Extracellular signal-regulated protein kinase 1/2 (ERK1/2) belongs to the mitogen-activated protein kinase (MAPK) family. A line of evidence has indicated that ERK1/2 is involved in the oxidative stress signaling pathways^[12]. Therefore, the present study was designed to investigate whether up-regulation of 14-3-3 protein and activation of both ERK1/2 and p38 MAPK are involved in HPP-induced cytoprotection.

2 Materials and methods

2.1 Reagents Cell culture reagents were purchased from Gibco. Anti-14-3-3 anti-phosphorylated p38 MAPK polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti- γ -tubulin monoclonal antibody, H₂O₂, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), PD98059, MPP⁺ and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma. Enzyme-linked immunosorbent assay (ELISA) kit for ERK1/2 was purchased from Kangchen BioTech, China.

2.2 Cell culture Rat pheochromocytoma PC12 cells were obtained from the China Center for Type Culture Collection (CCTCC). Cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) plus 10% horse serum and 5% fetal calf serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin, in humidified 5% CO₂ atmosphere at 37 °C. Cells were passaged every 2 or 3 d.

2.3 Experimental groups and protocols A known number (1×10^6 cells) of PC12 cells were randomly and homogeneously distributed into one of six groups as follows, and every group included two parallel wells for three replicate experiments on different preparations. (1) Control group: PC12 cells were incubated in DMEM without any drug treatment. (2) PD98059+Control group: PC12 cells were preincubated with 50 mmol/L PD98059 for 60 min and then performed as Control group. (3) MPP⁺ group: PC12 cells were exposed to 100 μ mol/L MPP⁺ for 24 h. (4) PD98059+MPP⁺ group: PC12 cells were preincubated with 50 mmol/L PD98059 for 60 min and then performed as MPP⁺ group. (5) Hydrogen peroxide preconditioning (HPP) group: PC12 cells were preconditioned with H₂O₂ (20 μ mol/L) exposure for 90 min followed by 24-h recovery, and subsequent exposure to 100 μ mol/L MPP⁺ for 24 h. (6) PD98059+HPP group: PC12 cells were preincubated with 50 mmol/L PD98059 for 60 min and then performed as HPP group. PD98059 was used to inhibit ERK1/2 phosphorylation.

2.4 Assay of cell viability Cell viability was determined by MTT assay, which is a colorimetric assay based on the ability of live but not dead cells to reduce a tetrazolium-based compound to a blue formazan product. The PC12 cells were seeded in 96-well plates at 1×10^5 cells/well. After treatment, cells were washed with warm phosphate-buffered saline (PBS) and incubated with 0.5 g/L MTT in PBS for 4 h at 37 °C. The reaction was stopped by addition of 150 μ L diphenylamine solution, and the absorbance of blue formazan derivative was measured at 570 nm using a spectrophotometer. The result of cell viability was expressed as a percent to the control value.

2.5 DAPI staining for assessment of apoptosis Chromosomal condensation and morphological changes in the nucleus were observed using the chromatin dye DAPI. The PC12 cells were fixed with 4% paraformaldehyde in 0.1 mol/L PBS for 10 min. After three rinses with PBS, the PC12 cells were stained with 2 mg/L DAPI at 37 °C for 15 min in the dark. Cells were washed once with methanol and covered with 10% PBS-glycerol. The morphology of cellular nuclei was observed by using a fluorescence microscope (Olympus, BX50-FLA, Japan) at an excitation wavelength of 350 nm. Cells with condensed nuclei or nuclear condensations were scored as apoptotic. Total 1×10^3 cells were counted in each group.

2.6 Western blot Protein preparation was done according to the standard method. Protein concentration of the supernatant was determined by the bicinchoninic acid (BCA) assay, and the protein supernatant (20 μ g/lane) was run on SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was then incubated for 1 h in PBS containing 5% fat-free dried milk, and then incubated with rabbit polyclonal antibodies to 14-3-3 (1:1 000), phosphorylated form of p38 MAPK (1:500), and mouse monoclonal antibody to γ -tubulin (1:10 000), respectively, at 4 °C overnight; followed by 2-h incubation with horseradish peroxidase conjugated goat anti-rabbit (1:5 000) or rat anti-mouse IgG (1:4 000). Protein bands

were detected with chemiluminescent reagent (ECL plus Western Blotting Detection System, Amersham, USA). The intensities of obtained bands were determined by a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, USA). Each densitometric value was expressed as mean $\pm$ SD.

2.7 Determination of ERK1/2 phosphorylation level in PC12 cells PC12 cells were seeded on 96-well plates at a density of 2 000 cells/well. After 24 h of incubation, the cells were treated as the protocols. The phosphorylation level of intracellular ERK1/2 was determined by using the ELISA kit for ERK1/2, following the manufacturer's instructions. The total amounts of ERK1/2 and phosphorylated ERK1/2 were estimated by this ELISA kit using anti-ERK1/2 and anti-phosphorylated ERK1/2 antibodies, respectively.

2.8 Statistical analysis Data are expressed as mean $\pm$ SD. Differences in means between groups were tested by one-way ANOVA. The relationship between the ERK1/2 activities and 14-3-3 protein expression was assessed by means of the *Pearson* coefficient (*r*), and the significance of the correlations was assessed with the Fisher *z* test^[9]. *P* < 0.05 was considered statistically significant.

3 Results

3.1 Effects of HPP on cell viability in PC12 cells treated with MPP⁺ Cell viability was usually used as an index of cell injury. As shown in Fig. 1, the viability of PC12 cells in the control and MPP⁺-treated cells was 93.4% and 51.6%, respectively. However, the cell viability in PD98059+MPP⁺ group was similar to that in MPP⁺ group (*P* > 0.05). In the HPP group, the viability (83.4%) of PC12 cells was significantly

Fig. 1 Effects of HPP on viability of PC12 cells subjected to MPP⁺ exposure. Data are expressed as mean $\pm$ SD. The values were obtained from six wells (4×10^4 cells per well) of 96-well plate per experiment, and averaged from four independent experiments. HPP: hydrogen peroxide preconditioning. ***P* < 0.01 vs Control group, **P* < 0.01 vs MPP⁺ group, #*P* < 0.05 vs HPP group.

cantly increased compared with that in MPP⁺ group (*P* < 0.01). This suggested that HPP could induce protective effects on PC12 cells. However, when given 60 min prior to HPP, PD98059 (an inhibitor of ERK1/2) abolished the protection induced by HPP. To verify that the PD98059-mediated effects are not a consequence of cytotoxicity, PC12 cells were pretreated with PD98059 alone (PD98059+Control group). Our result showed that PD98059 alone had no effect on the viability of PC12 cells.

3.2 Effects of hydrogen peroxide preconditioning on MPP⁺-induced PC12 cell apoptosis

In order to examine the morphological changes of apoptotic cells induced by MPP⁺, PC12 cells were stained with DAPI. Normal cells have a round or ovoid shape (light blue fluorescence). Apoptotic cells display condensation and fragmentation of the nuclei (sapphirine fluorescence, DAPI-staining positive). As shown in Fig. 2A, very few DAPI-staining positive cells were detected among PC12 cells from Control group and PD98059+Control group. Numerous cells from MPP⁺ group and PD98059+MPP⁺ group were shown chromatin condensation and fragmentation of the nuclei. In contrast, the number of DAPI-staining positive cells was significantly reduced in the PC12 cells from HPP group. However, in PD98059+HPP group, the number of DAPI positive cells increased markedly compared with that in HPP group (*P* < 0.01) and similar to that in MPP⁺ group (*P* > 0.05). Quantitative data showed that (51.3 $\pm$ 6.6)% of MPP⁺-treated PC12 cells were apoptotic, whereas only (24.9 $\pm$ 4.3)% cells underwent apoptosis in the HPP group. But PD98059 blocked the anti-apoptotic effect of HPP, shown as percentage of apoptotic cells, which was (48.2 $\pm$ 6.8)% in the PD98059+HPP group. The percentages of apoptotic cells in the Control group, PD98059+Control group, and PD98059+MPP⁺ group were (7.2 $\pm$ 2.1)%, (7.6 $\pm$ 2.4)%, and (53.6 $\pm$ 6.2)%, respectively (Fig. 2B).

3.3 Effect of hydrogen peroxide preconditioning on 14-3-3 protein expression

The induction of 14-3-3 protein by HPP was verified by Western blot analysis. As shown in Fig. 3A and B, the basic expression of 14-3-3 protein was present in Control group, and there was no significant difference in 14-3-3 protein expression between Control group and PD98059+Control group. The expression of 14-3-3 protein in MPP⁺ group decreased by 39% compared with that in Control group (*P* < 0.05). However, the expression of 14-3-3 protein increased by 98% in HPP group compared with that in

A

B

Fig. 2 Nuclear staining in PC12 cells with DAPI. A: Representative photomicrographs of DAPI-staining apoptotic PC12 cells from Control, PD98059+Control, MPP⁺, PD98059+MPP⁺, HPP, and PD98059+HPP groups. Scale bar, 20 μ m. B: Quantitative analysis of apoptotic PC12 cells. Results are expressed as percentage of DAPI-staining nuclear condensation and fragmentation in PC12 cells. Total 1×10^3 cells were counted in each group. Values are expressed as mean $\pm$ SD of four independent experiments. ** $P < 0.01$ vs Control group, ## $P < 0.01$ vs MPP⁺ group, ss $P < 0.01$ vs HPP group.

A

B

Fig. 3 Western blot analysis of 14-3-3 protein expression in PC12 cells. A: Representative Western blot is shown. B: Relative levels of 14-3-3 protein as assessed by densitometry. Quantitation was normalized to the value of γ -tubulin protein expression. Values are expressed as mean $\pm$ SD of four independent experiments ($n = 4$). * $P < 0.05$ vs Control group, ** $P < 0.01$ vs MPP⁺ group, # $P < 0.05$ vs HPP group.

MPP⁺ group ($P < 0.01$); while 14-3-3 protein in PD98059+APC group decreased dramatically when compared with HPP group ($P < 0.01$), and was similar to that in MPP⁺ group ($P > 0.05$). 14-3-3 Protein in PD98059+MPP⁺ group was also similar to that in MPP⁺ group ($P > 0.05$). This clearly demonstrates that PD98059 can suppress the up-regulation of 14-3-3 protein induced by HPP.

3.4 Effects of hydrogen peroxide preconditioning on the activity of ERK1/2 The phosphorylation of ERK1/2 was evaluated by ELISA. As shown in Fig. 4, the level of phosphorylated ERK1/2 between Control group and PD98059+Control group had no significant difference ($P > 0.05$), while it was enhanced by 39% in MPP⁺ group compared with that in Control group. The enhancement of phosphorylated ERK1/2 in HPP group was about 2.4 times greater than that in Control group. When given 60 min prior to HPP, PD98059 abolished the activation of ERK1/2 induced by HPP, compared with that in HPP group ($P < 0.01$). However, PD98059 alone had no obvious effects on the activity of ERK1/2 in PC12 cells treated with MPP⁺.

Fig. 4 Phosphorylation levels of ERK1/2 after HPP treatment were estimated by ELISA. Relative phosphorylation levels of ERK1/2 were estimated by dividing the amount of phosphorylated ERK1/2 by the total amount of ERK1/2. Values are expressed as mean±SD of four independent experiments ($n = 4$). * $P < 0.05$ vs Control group, ** $P < 0.01$ vs MPP⁺ group, # $P < 0.01$ vs HPP group.

3.5 Effects of hydrogen peroxide preconditioning on p38 MAPK expression Figure 5 shows the alteration of p38 MAPK protein expression in PC12 cells. The expression of p38 MAPK in the PC12 cells of Control group and PD98059+Control group did not show any significant difference ($P > 0.05$). The MPP⁺ group showed a 45% decrease of p38 MAPK expression compared with that in Control group ($P < 0.05$). HPP induced about 3-time enhancement of p38 MAPK expression as compared with MPP⁺ group ($P < 0.01$). When

Fig. 5 Western blot analysis of p38 MAPK protein expression in PC12 cells. A: Representative Western blot is shown. B: Relative levels of phosphorylated p38 MAPK protein as assessed by densitometry. Quantization was normalized to value obtained from γ-tubulin protein expression. Values are expressed as mean±SD of four independent experiments ($n = 4$). * $P < 0.05$ vs Control group, ** $P < 0.01$ vs MPP⁺ group, # $P < 0.01$ vs HPP group.

given 60 min prior to HPP, PD98059 inhibited the elevation of p38 MAPK induced by HPP as compared with HPP group ($P < 0.01$). However, PD98059 alone had no obvious effect on the expression of p38 MAPK in PC12 cells undergoing MPP⁺ treatment.

3.6 Relationship between ERK1/2 activation and 14-3-3 protein expression A significant ($P < 0.01$) and positive correlation ($r = 0.923$) was found between the activation of ERK1/2 and expression of 14-3-3 protein in PC12 cells. Furthermore, a significant negative correlation was found between 14-3-3 protein expression and PC12 cell apoptosis ($r = -0.941$, $P < 0.01$).

4 Discussion

In the present study, the most important observation was that HPP increased 14-3-3 protein expression in PC12 cells, in parallel with cytoprotection against MPP⁺ toxicity. Furthermore, the study indicates that such expression changes of 14-3-3 may be regulated through the ERK1/2 signaling pathway.

The protection of preconditioning, which is thought to be a universal protective mechanism in the living body, was reported first in 1986 by Murry *et al.*^[13] and was further investigated in different animal species and organs, but most

of these investigations were performed in myocardial cell cultures and heart tissue. Recently, some reports showed that H₂O₂ preconditioning could protect PC12 cells against apoptosis induced by H₂O₂^[4] and dopamine^[5]. In the present study, we found that H₂O₂ preconditioning could increase the viability and inhibit the apoptosis induced by MPP⁺ in PC12 cells. Our data further demonstrate that HPP has a protective effect on PC12 cells.

Although the protective mechanisms of HPP are incompletely understood, some evidences suggest that the protection may be conferred by *de novo* translation of some endogenous protective proteins^[14]. In this study, we found that 14-3-3 protein expression was significantly up-regulated after HPP treatment. Moreover, this effect was accompanied by increase of cell viability and inhibition of MPP⁺-induced apoptosis. These findings suggest that 14-3-3 protein may be involved in the protection of HPP as an endogenous protective protein.

14-3-3 Protein is an endogenous brain-specific protein and mediates an essential anti-apoptotic signal for cell survival^[15]. 14-3-3 Proteins display anti-apoptotic characteristics by inactivating the pro-apoptotic molecules including BAD, FKHRL-1, ASK1 and A20^[7]. In harmony with this, we have previously found that overexpression of 14-3-3 inhibited the apoptosis of PC12 cells induced by MPP⁺^[11]. Additionally, Chen *et al.*^[9] also have shown that anoxic preconditioning up-regulates the expression of 14-3-3 protein in neonatal rat cardiomyocytes and protects cardiomyocytes from apoptosis induced by anoxia/reoxygenation injury. These reports further suggest that 14-3-3 protein is associated with the cellular resistance to apoptosis. In the present study, we found that 14-3-3 protein expression was negatively correlated with PC12 cell apoptosis ($r = -0.941$, $P < 0.01$), suggesting that the protection of HPP may be associated with the anti-apoptosis of 14-3-3 protein.

H₂O₂, which has emerged as an important signaling molecule involved in a variety of cell types, acts in the mitogenic or survival signaling as well as in the stress signaling^[16,17]. ERK1/2 pathway is the best studied of all MAPK subfamily pathways, and it has been implicated in cell survival in many cells^[18]. In the present study, we found that, when preconditioned with H₂O₂, PC12 cells from HPP group were more resistant to MPP⁺ and had much stronger phosphorylation of ERK1/2 than those from control, and these effects were at-

tenuated by PD98059, an inhibitor of ERK1/2 phosphorylation. These observations suggest that the activation of ERK1/2 may be an important component involved in the HPP-induced protection on cells. Furthermore, we found that the phosphorylation of ERK1/2 was accompanied by the up-regulation of phosphorylated p38 MAPK and 14-3-3 protein, and the 14-3-3 protein expression was positively correlated to the phosphorylation of ERK1/2 ($r = 0.923$, $P < 0.01$). Nevertheless, inhibition of the ERK1/2 with PD98059 abolished the phosphorylation of p38 MAPK and up-regulation of 14-3-3 protein induced by HPP in PC12 cells. These findings indicate that HPP-induced up-regulation of 14-3-3 protein may be regulated through ERK1/2 signaling pathway. On the other hand, our findings also indicate that p38 MAPK may provide a potential link between ERK1/2 activation and 14-3-3 protein expression, which are responsible for the protective function of HPP. A number of studies have demonstrated that activation of p38 MAPK is implicated in the neuronal damages associated with ischemia, neurodegenerative disease, and excitotoxicity^[19]. However, our results provided possible evidence to indicate that p38 MAPK activation is involved in the protection of HPP. The mechanisms deserve further study.

In conclusion, the present study provided unequivocal evidence that HPP could act through 14-3-3 protein up-regulation. Moreover, we have also shown that the up-regulation of 14-3-3 protein induced by HPP might involve the phosphorylations of ERK1/2 and p38 MAPK. However, detailed definition of the relations between the ERK and 14-3-3 protein and the role of p38 MAPK in 14-3-3 mediated protection of HPP will require further investigation.

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H_2O_2 预处理通过 ERK1/2 和 p38 MAPK 信号通路上调 14-3-3 蛋白在 PC12 细胞中的表达

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摘要: 目的 探讨 H_2O_2 预处理对 1-甲基-4-苯基吡啶离子(1-methyl-4-phenylpyridinium, MPP^+)诱导 PC12 细胞毒性损伤的保护作用及其可能的机制。方法 分别用 3-(4,5-二甲基噻唑-2)-2,5-二苯基四氮唑溴盐[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT]和 4,6-二氨基-2-苯基吲哚(4',6'-diamidino-2-phenylindole, DAPI)染色技术检测 PC12 细胞的活力及细胞凋亡核形态改变; Western blot 检测 PC12 细胞中 14-3-3 蛋白、磷酸化 p38 MAPK 的水平。酶联免疫吸附实验(Enzyme-linked immunosorbent assay, ELISA)试剂盒检测 ERK1/2 磷酸化水平。结果 MPP^+ 处理 PC12 细胞 24 h 使细胞活力显著下降(51.6%), DAPI 染色显示(51.3±6.6)%的 PC12 细胞呈现核固缩等细胞凋亡形态改变; H_2O_2 预处理能显著增加 PC12 细胞活力(83.4%), 减少凋亡细胞数[(24.9±4.3)%], 说明 H_2O_2 预处理能对抗 MPP^+ 毒性, 对 PC12 细胞具有保护作用。Western blot 结果显示, H_2O_2 预处理在减少 PC12 细胞凋亡的同时伴随有 14-3-3 蛋白及磷酸化 p38 MAPK 表达上调; ELISA 实验显示 ERK1/2 磷酸化水平显著增加。相关分析显示 14-3-3 蛋白表达的上调与 ERK1/2 磷酸化水平呈正相关($r=0.923$, $P<0.01$), 而且 PD98059 抑制 ERK1/2 磷酸化后, H_2O_2 预处理诱导 14-3-3 蛋白表达上调的作用消失。结论 H_2O_2 预处理能对抗 MPP^+ 对 PC12 细胞的毒性作用, 这种保护作用与细胞内 14-3-3 蛋白表达上调有关; 而 14-3-3 蛋白表达上调与 ERK1/2 和 p38 MAPK 信号通路的激活有关。

关键词: H_2O_2 预处理; 14-3-3 蛋白; ERK1/2; p38 有丝分裂原激活蛋白激酶; PC12 细胞