Muscarinic receptor activation protects against paraquat-induced apoptosis in human neuroblastoma SH-SY5Y cells

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Abstract

Paraquat (PQ), a widely used herbicide, has been shown to exhibit neurotoxicity partly through its ability to induce oxidative stress and apoptosis. Muscarinic receptor activation was reported to protect cell against oxidative stress. Therefore, the present study examined whether muscarinic receptor activation provided protection against PQ-induced apoptosis. The results showed that PQ induced cytotoxicity in human neuroblastoma SH-SY5Y cells. PQ caused augmentation in proteolysis of poly-ADP ribose-polymerase (PARP) which is a substrate of caspase 3/6 indicating apoptosis activation by PQ. Moreover, levels of p53 which is a protein involved in apoptotic cell death were increased following PQ treatment. Treatment with muscarinic receptor agonists, carbachol and oxotremorine-M attenuated PQ-induced PARP proteolysis and elevation of p53. Interestingly, the attenuation actions of carbachol and oxotremorine-M were abolished by pretreatment with a muscarinic receptor antagonist, atropine. These results indicate that muscarinic receptor activation provides protection against PQ-induced apoptotic cell death.

Key words: Apoptosis, Muscarinic receptor, Neurotoxicity, Paraquat

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การกระตุ้นตัวรับมัสคารินิกป้องกันการตายแบบอะพอพโตสิสที่เกิดจากพาราควอทในเซลล์มะเร็งสมอง SH-SY5Y

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บทคัดย่อ

พาราควอท (PQ) เป็นสารกำจัดวัชพืชที่ใช้กันอย่างแพร่หลาย เป็นพิษต่อระบบประสาทโดยผ่านทางการก่อให้เกิดอนุมูลอิสระ ความเครียดออกซิเดชั่นและการตายแบบอะพอพโตสิส และมีรายงานว่า การกระตุ้นตัวรับมัสคารินิก สามารถป้องกันเซลล์จากผลร้ายของอนุมูลอิสระได้ ดังนั้นการศึกษานี้เพื่อทดสอบว่าการกระตุ้นตัวรับมัสคารินิกสามารถป้องกันเซลล์จากการตายแบบอะพอพโตสิสซึ่งเหนี่ยวนำโดย PQ ได้หรือไม่ ผลการศึกษาพบว่า PQ สามารถทำให้เซลล์มะเร็งสมอง SH-SY5Y ตายได้ และทำให้เกิด proteolysis ของ poly-ADP ribose-polymerase (PARP) ซึ่งเป็นสารตั้งต้นของเอนไซม์ caspase 3/6 แสดงให้เห็นว่า PQ ทำให้เกิดการตายแบบอะพอพโตสิสในเซลล์มะเร็งสมอง SH-SY5Y นอกจากนี้ยังพบว่าระดับของ p53 ซึ่งเป็นโปรตีนที่มีส่วนร่วมในกลไกการตายแบบอะพอพโตสิสนั้นเพิ่มขึ้น เมื่อเซลล์ได้รับสารกระตุ้นตัวรับมัสคารินิก (carbachol และ oxotremorine-M) ก่อนที่จะได้รับ PQ ทำให้ PQ กระตุ้นการตายแบบอะพอพโตสิสและลดการเพิ่มขึ้นของ p53 นอกจากนี้ยังพบว่าความสามารถในการป้องกันการตายจาก PQ นี้จะหมดไป เมื่อเซลล์ได้รับสารต้านการกระตุ้นตัวรับมัสคารินิก (atropine) ร่วมด้วย ผลการศึกษานี้แสดงให้เห็นว่า การกระตุ้นตัวรับมัสคารินิกสามารถป้องกันเซลล์จากการตายแบบอะพอพโตสิสซึ่งเหนี่ยวนำโดย PQ ได้

คำสำคัญ: อะพอพโตสิส ตัวรับมัสคารินิก ความเป็นพิษ ระบบประสาท พาราควอท

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Introduction

Paraquat (PQ) or "N,N′-dimethyl-4,4′-bipyridinium dichloride" is a pesticide widely used in agricultural processes. Epidemiological studies have demonstrated the association of PQ exposure to development of Parkinson's disease[1-4]. However, the mechanisms underlying PQ-induced Parkinson's disease are not fully understood. It is generally believed that the generation of reactive oxygen species (ROS) and subsequent induction of apoptosis play critical role in PQ neurotoxicity. Mechanistically, PQ-induced apoptosis was reported to involve mitochondria disruption leading to cytochrome c release which in turn leading to activation of caspase-3/9 and PARP proteolysis[5-8]. Moreover, it has been reported that PQ-induced p53 accumulation plays an important role in initiation of mitochondria apoptosis[8].

Muscarinic receptor activation has been shown to protect neurons from oxidative stress-induced apoptosis[9-12]. Considering the important of ROS generation in induction of apoptosis by PQ, we investigated whether muscarinic receptor activation protected against PQ-induced neurotoxicity.

Materials and methods

Cell culture: Human neuroblastoma SH-SY5Y cells expressing endogenous muscarinic receptors were grown in mixture of MEM and Ham's F12 media (1:1) (Gibco) supplemented with 10% fetal bovine serum (FBS, JR Scietific, Inc), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). Cells were maintained in humidified chamber at temperature 37°C and 5% carbon dioxide of 95% atmospheric air.

Treatments: Cells (70-80% confluence) were treated with PQ for 24 hours. Where indicated, 300 µM of carbachol or oxotremorine-M was added for 30 minutes prior to 24 hour PQ treatment. To block muscarinic receptor activation, cells were treated with 10 µM of atropine for 15 minutes before the addition of muscarinic receptor agonist (carbachol or oxotremorine-M). Sterile water was used as vehicle control.

Lactase dehydrogenase (LDH) cytotoxicity assay: Cytotoxicity was assayed by LDH release using LDH cytotoxicity detection kit (Roche Diagnostics) according to protocol described by manufacturer. Absorbance was measured at 490 nm with reference wavelength at 600 nm by using microplate reader (SpectraMax, Molecular Devices). Cytotoxicity was reported as % cytotoxicity above cytotoxicity in the control sample.

Cell lysate preparation: At the end of treatment, cells were scraped off, transferred to 15 ml conical tube, and centrifuged at 1500 rpm, 4°C for 5 minutes. Cell pellets

were washed with cold PBS. The cell pellets were lysed in lysis buffer containing 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP40, protease inhibitor cocktail (Calbiochem), 0.5mMPMSF, 1 mM Na_3VO_4 and 20 mM NaF. Then cell lysates were sonicated, and centrifuged at 16,000 x g for 15 min at 4°C. The supernatant was transferred to new labeled 1.5 ml tube and kept at -80˚C until analysis. Protein concentrations were determined using the Bradford reagent (BioRad).

Immunoblotting analysis: Protein samples were mixed with loading dye containing 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β-mercaptoethanol, and boiled for 5 minutes. The protein samples were resolved on SDS-PAGE, and transferred to nitrocellulose membrane. The membrane was incubated in blocking buffer of 5% nonfat dried milk in TBST (20 mM Tris-buffer saline, pH 7.4 with 0.1% Tween) for 1 hour at room temperature with gentle shaking. Thereafter, the membrane was incubated with primary antibody in 5% nonfat dried milk in TBST for overnight at 4˚C with gentle shaking. The membrane was washed with TBST for three times followed by incubation with appropriate secondary antibody linked with horseradish peroxidase for 2 hours at room temperature with gentle shaking. Then the membrane was washed and incubated in enhanced chemiluninescence (ECL) to visualize protein bands. The immunoreactive protein band was detected using X-ray film. Antibodies against human PARP (1:1,000) (BD), p53 (1:2,000) (Santa Cruz) and β-actin (1:20,000) (Sigma-Aldrich) were used. Protein bands were scanned (ImageScanner III, GE healthcare), and quantified using Image Quant TL software (GE Healthcare). Levels of PARP and p53 were normalized to β-actin measured on the same blot. Levels of protein were calculated relative to control cells, and expressed as fold of control.

Statistics: At least three independent experiments were completed for each treatment. All data are mean ± SEM. Statistical significances were assessed by ANOVA followed by post-hoc LSD analysis.

Results and discussion

PQ induces cytotoxicity in human neuroblastoma SH-SY5Y cells

To determine PQ toxicity in human neuroblastoma SH-SY5Y cells, cells were treated with various concentrations of PQ (0-2,000 μM) for 24 hours. Cytotoxicity was assessed by determination of lactate dehydrogenase (LDH) release assay. The results demonstrated that PQ caused dose dependent cytotoxicity in SH-SY5Y cells (Fig. 1) which was consistent with previous

report[5,8,14]. A significant increased cytotoxicity above control level was observed at 1000 and 2000 μM of PQ (Fig.1) suggesting that at these concentrations, PQ caused prominent neurotoxicity. Therefore, we used PQ at 1000 and 2000 μM in further studies.

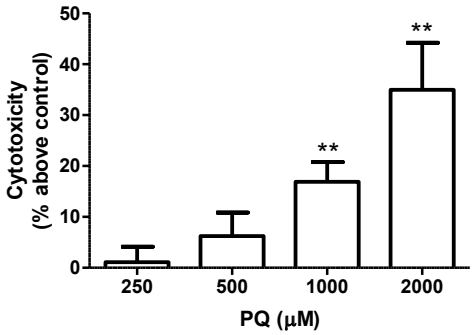

Figure 1 PQ induces cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were treated with PQ for 24 hours. Cytotoxicity was assessed by using LDH cytotoxicity detection kit (Roche Diagnotics). Data are mean ± SEM. $^{**}p > 0.01$.

PQ induces apoptosis in SH-SY5Y cells

We examined whether PQ-mediated cell death through apoptosis pathway, SH-SY5Y cells were treated with PQ for 24 hours, and apoptosis was determined by activation of the executioner caspases, caspase-3/6 by measurement of proteolysis of PARP which is a substrate of caspase-3/6[13]. Immunoblot analysis revealed that PQ caused prominent increase in PARP proteolysis (Figs. 2A and B). The results indicate that exposure to PQ activates apoptosis in SH-SY5Y cells. Our results were in agreement with previous reports that PQ induces apoptosis in neuronal cells[5,12,14]. Moreover, levels of p53 were drastically elevated by PQ treatment in SH-SY5Y cells (Figs. 3A and B). It is well known that induction of p53 is an initiation for apoptosis induced by various stimuli including ROS[15]. Additionally, p53 has been shown to be involved in PQ-induced apoptosis[5,12]. Hence, our finding that p53 was elevated by PQ suggests PQ may initiate apoptosis in SH-SY5Y cells through induction of p53.

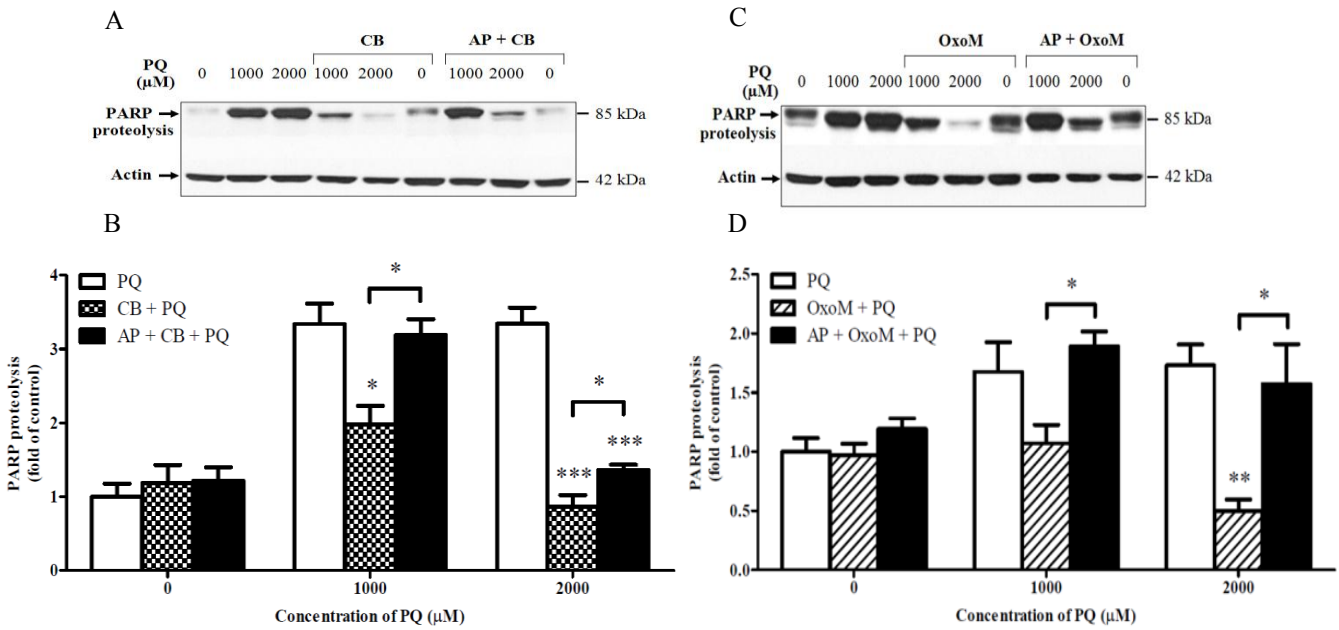

Figure 2 Muscarinic receptor activation attenuates PQ-induced PARP proteolysis and atropine, a muscarinic receptor antagonist, blocks this attenuation. SH-SY5Y were treated with or without 10 μM atropine (AP) for 15 minutes, followed by 30 minute treatment of muscarinic receptor agonist; 300 μM carbachol or oxotremorine-M prior to incubation with PQ for 24 hours. PARP proteolysis was measured by immunoblot analysis. Representative blots demonstrating the protective effect of carbachol (CB) (A) and oxotremorine-M (OxoM) (C) against PQ-induced PARP proteolysis, and the blockade of their protections by atropine (AP) were shown. Quantitative analyses of CB and OxoM protective effects were shown in B and D, respectively.

Muscarinic receptor agonists, carbachol and oxotremorine-M attenuate PQ-induced apoptosis

To test whether muscarinic receptor activation affected PQ-induced apoptosis, two muscarinic receptors agonists, carbachol and oxotremorine-M were used. SH-SY5Y cells were treated with 300 μM of carbachol or oxotremorine-M for 30 minutes followed by treatment of PQ for 24 hours. The results showed that carbachol pretreatment caused significant attenuation of PQ-induced PARP proteolysis (Figs. 2A and B). Similarly, the attenuation of PQ-induced PARP cleavage was also observed in oxotremorine-M pretreatment (Figs. 2C and D). These indicate that muscarinic receptor activation protects against PQ-induced apoptosis. Moreover, carbachol or oxotremorine-M pretreatment significantly decreased the induction of p53 by 24 hour treatment with 2,000 μM PQ but not with 1,000 μM PQ (Fig. 3). Supporting by the report that muscarinic receptor activation

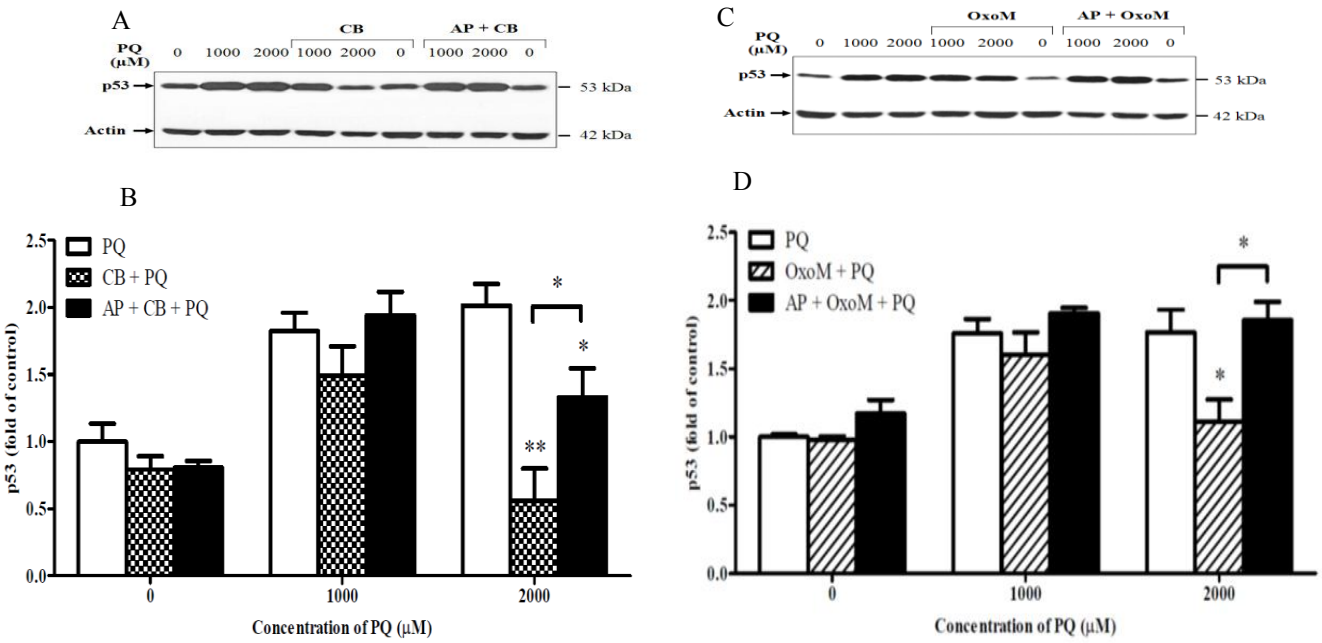

Figure 3 Effect of muscarinic rector activation on PQ-induced p53 induction. SH-SY5Y were treated with or without 10 μM atropine (AP) for 15 minutes, followed by 30 minute treatment of muscarinic receptor agonist; 300 μM carbachol or oxotremorine-M prior to incubation with PQ for 24 hours. Level of p53 was measured by immunoblot analysis. Representative blots demonstrating the effect of carbachol (CB) (A) and oxotremorine-M (OxoM) (C), and combination of CB or OxoM with atropine (AP) on PQ-induced p53 accumulation were shown. Quantitative analyses of CB and OxoM effects on p53 accumulation were shown in B and D, respectively.

exerted its anti-apoptosis against oxidative stress without affecting p53 accumulation[9], we speculate that its protection against PQ-induced apoptosis is unlikely to be due to inhibition of p53.

Muscarinic receptor activation protects against PQ-induced apoptosis

To confirm that the protective effect against PQ-induced apoptosis by carbachol and oxotremorine-M was through muscarinic receptor stimulation, a specific muscarinic receptor antagonist, atropine was used to block muscarinic receptor stimulation. The results showed that the apoptotic protective effect of carbachol and oxotremorine-M against PQ was prevented by atropine pretreatment as it caused increases in levels of PARP proteolysis when compared the levels in cotreatment of PQ with carbachol or oxotremorine-M (Fig. 2). These results confirm that muscarinic receptor agonists, carbachol and oxotremorine-M provide protection against PQ-induced apoptosis in SH-SY5Y cells through activation of muscarinic receptor. It is interesting to note that atropine caused a complete blockade of oxotremorine-M protection against PQ-induced PARP

proteolysis (Figs. 2C and D) while it partially blocked the protection by carbachol (Figs. 2A and B). These finding may imply an influence of nicotinic receptor activation in carbachol anti-apoptotic action against PQ since carbachol stimulates not only muscarinic cholinergic receptors but also nicotinic cholinergic receptors. There are 5 subtypes of muscarinic receptor. Our finding that oxotremorine-M which is a selective muscarinic receptor sybtype 3 (M3R) prevents PQ-induced apoptosis implicate the anti-apoptotic action of M3R. Whether other muscarinic receptor subtypes possess anti-apoptotic action remains to be investigated.

In conclusion, the present study demonstrates that muscarinic receptor activation protects against PQ-induced apoptosis in human neuroblastoma SH-SY5Y cells.

References

1. Baltazar MT, Dinis-Oliveira RJ, de Lourdes Bastos M, *et al*. Pesticides exposure as etiological factors of Parkinson's disease and other neurodegenerative diseases-a mechanistic approach. *Toxicol lett* 2014; 230: 85-103.
2. Berry C, La Vecchia C, Nicotera P. Paraquat and Parkinson's disease. *Cell Death Diff* 2010; 17: 1115-25.
3. Moretto A, Colosio C. The role of pesticide exposure in the genesis of Parkinson's disease: epidemiological studies and experimental data. *Toxicology* 2013; 307: 24-34.
4. Spivey A. Rotenone and paraquat linked to Parkinson's disease: human exposure study supports years of animal studies. *Env Health Pers* 2011; 119: A259.
5. Chang X, Lu W, Dou T, *et al.* Paraquat inhibits cell viability via enhanced oxidative stress and apoptosis in human neural progenitor cells. *Chem Biol Interac* 2013; 206: 248-55.
6. Cocheme HM, Murphy MP. Complex I is the major site of mitochondrial superoxide production by paraquat. *J Biol Chem* 2008; 283: 1786-98.
7. Wang X, Luo F, Zhao H. Paraquat-induced reactive oxygen species inhibit neutrophil apoptosis via a p38 MAPK/NF-kappaB-IL-6/TNF-alpha positive-feedback circuit. *PloS one* 2014; 9: e93837.
8. Yang W, Tiffany-Castiglioni E. Paraquat-induced apoptosis in human neuroblastoma SH-SY5Y cells: involvement of p53 and mitochondria. *J Toxicol Env Health Part A* 2008; 71: 289-99.
9. De Sarno P, Shestopal SA, King TD, *et al.* Muscarinic receptor activation protects cells from apoptotic effects of DNA damage, oxidative stress, and mitochondrial inhibition. *J Biol Chem* 2003; 278: 11086-93.

10. Giordano G, Li L, White CC, *et al.* Muscarinic receptors prevent oxidative stress-mediated apoptosis induced by domoic acid in mouse cerebellar granule cells. *J Neurochem* 2009; 109: 525-38.
11. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007; 35: 495-516.
12. Sun L, Zang WJ, Wang H, *et al.* Acetylcholine Promotes ROS Detoxification against hypoxia/ reoxygenation-induced oxidative stress through FoxO3a/PGC-1alpha dependent superoxide dismutase. *Cell Physiol Biochem* 2014; 34: 1614-25.
13. Yang B, Lin H, Xu C, *et al.* Choline produces cytoprotective effects against ischemic myocardial injuries: evidence for the role of cardiac m3 subtype muscarinic acetylcholine receptors. *Cell Physiol Biochem* 2005; 16: 163-74.
14. Fujimori K, Fukuhara A, Inui T, *et al.* Prevention of paraquat-induced apoptosis in human neuronal SH-SY5Y cells by lipocalin-type prostaglandin D synthase. *J Neurochem* 2012; 120: 279-91.
15. Vousden KH, Prives C. Blinded by the light: the growing complexity of p53. *Cell* 2009;137: 413-31.