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Detrimental Effects of Paraquat on Astrocytes-Regulating Synaptic Functions

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Abstract

Paraquat (PQ) exposure is known as a risk factor for developing neurodegenerative diseases. Astrocytes are implicated and affected in neurodegenerative diseases and brain injuries, so it is suspected that PQ may impose detrimental effects on astrocytes function. Here, we present a study of the effects of PQ on synaptic function of astrocytes. Human astrocytes (HA1800) were separately treated with PQ at 200, 400, and 800 μmol/L for 12, 24, and 48 hours, respectively. The concentrations of membrane cholesterol, intracellular glutamate, and adenosine triphosphate (ATP) were assessed by measuring changes with Kits after PQ treatment. Expression levels of glial fibrillary acidic protein (GFAP) and apolipoprotein E (ApoE) were detected using Western blot method. The results indicated that astrocytes were highly susceptible to PQ showing astrogliosis characterized by enhanced GFAP expression ($P < .05$). The decreased cholesterol level and ApoE expression were observed in PQ-treated astrocytes and so were the decreased levels of glutamates and ATP in PQ-treated astrocytes. These results suggested that PQ exerted the detrimental effects on synaptic function of astrocytes, which may be involved in the progressive process of neurodegenerative disorders.

Keywords

paraquat, astrocytes, synaptic function, neurodegeneration

Introduction

Paraquat (1,1-dimethyl-4, 4-bipyridium dichloride; PQ), a nonselective herbicide, caught the attention of researchers because its molecular structure is similar to the neurotoxin, 1-methyl-4-phenylpyridinium ion, the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine. Chronic exposure to PQ was suspected to cause neurodegeneration in human, which has been experimentally tested in a large number of animal studies, although few investigators argued the neurodegenerative effects of PQ.[1,2] Some studies conducted in rodent models indicated that PQ caused neurotoxicity through the generation of reactive oxygen species (ROS).[3,4] Paraquat can promote intracellular generation of ROS via 3 distinct pathways, including reduction in PQ by NADPH-cytochrome P450 reductase and subsequent redox cycle, inhibition of mitochondrial electron transport chain, and interaction with other enzymes (such as nitric oxide synthase), all of which finally result in lipid peroxidation, cell damage, and death.[5-7] These are considered as main mechanisms of PQ neurotoxicity, especially the induced damage or death of nigral dopaminergic neurons.[7] In addition, there were supporting evidences that microglia activation occurs following PQ treatment and inflammatory cytokines are secreted, such as tumor necrosis factor α (TNF-α), interleukin 6, and interleukin 1β, and so on in agreement with our early study.[8-10] It was also reported that PQ specifically suppressed postsynaptic a-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor–mediated evoked excitatory postsynaptic currents in dopaminergic neurons of substantia nigra pars compacta, which may contribute to the pathogenesis of PD.[11] The exact mechanisms of PQ-induced neurotoxicity are still in debate. An understanding of the molecular basis of PQ-induced neurotoxicity would

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provide valuable insights into neurodegenerative processes in mammalian brain.

Under normal physiological conditions, astrocytes, accounting for more than half of the cells in the human brain, play key roles in the maintenance of brain homeostasis as well as formation, maturation, stabilization, and elimination of synapses. Also, astrocytes have the ability to control synaptogenesis and modulate synaptic activity and function.[12,13] Although astrocytes are implicated and affected in neurodegenerative diseases and brain injuries, their altered function may contribute to further spread of neurodegenerative changes.[14-16] The activation of astrocytes and their death may lead to neuronal loss, although the exact role of astrocytes in neurodegeneration is unknown. The aberrant synaptic function contributes to the synaptic deficits and is associated with the pathological process of neurodegenerative disorders and also the changes in synaptic activity occur prior to the pathological changes in neurons.[17] Thus, the changes in astrocytes condition can influence the information processing at synapses. It has been reported that PQ exerted the effect on synaptic growth and development of neurons through autophagy.[18] However, whether and how PQ affects synaptic function of astrocytes has not yet been thoroughly investigated. Here, we study the effects of PQ on astrocytes-regulating synaptic function and the ability of astrocytes to induce synapse formation and modify synaptic transmission in order to provide the evidences for exploring the possible neurotoxicity mechanisms of PQ at molecular levels.

Materials and Methods

Chemicals and Reagents

Paraquat (paraquat dichloride hydrate, 99% of purity) was obtained from Sigma Co, Ltd (St. Louis, MO). Bicinchoninic acid (BCA) Protein Quantification Kit and Membrane Protein Extraction Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The cholesterol, adenosine triphosphate (ATP), and glutamate Quantification Kit were purchased from Nanjing Institute of Biological Engineering (Nanjing, China). The polyclonal antibodies of glial fibrillary acidic protein (GFAP), apolipoprotein E (ApoE), β-actin, and alkaline phosphatase-conjugated secondary antibody were acquired from Wan lei Biological Technology Co, Ltd (Shenyang, China). All cell culture products were purchased from Gibco (San Diego, CA), except fetal bovine serum which was from NQBB (Sydney, Australia).

Paraquat stock solution (10 mmol/L) was prepared by dissolving PQ in distilled water and then kept at 4°C away from light. Before use, PQ stock solution was diluted into the corresponding application solution with culture medium.

Cell Culture and Treatment

Human astrocytes (HA1800) were cultured in the flask without coating in a humidified 5% CO_2 atmosphere in RPMI1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were seeded at a density of 2×10^4 cells/cm^2 onto the plastic flasks. When cells were about 80% confluent, they were treated with PQ for 12 hours, 24 hours, and 48 hours, respectively. The control cells were treated with only medium at the same time. After processing for the indicated times, the cells were collected by trypsin/EDTA digestion or cells scraper.

Determination of Membrane Cholesterol

Briefly, the equal amounts of cells were cracked with 1% Triton X-100 until cells were completely broken. The lysates were then added to 96-well plate with 6 wells per group and were treated according to the instruction of Cholesterol Quantification Kit. Absorbance values were determined using colorimetric spectrophotometry at 510 nm, and the concentration of cholesterol was calculated.

Determination of Neurotransmitters

Before the determination, the amount of cells in each group was adjusted to be consistent. Briefly, for astrocytic ATP test, the cell samples were broken up by homogenate in the boiling water, and the cell suspensions were heated in a boiling water bath for 10 minutes, then mixed, and lifted for 1 minute. As for glutamate test, the cells were broken with 1% Triton X-100. The suspension was treated according to the instruction of ATP and glutamate Quantification Kit. Absorbance values were determined with spectrophotometer at 636 nm, 0.5 cm cuvette and 340 nm and 1 cm cuvette, respectively. The concentration of ATP and glutamate was calculated.

Extraction of Membrane Protein

About 3 to 4×10^7 cells were collected using cells scraper and washed with phosphate-buffered saline solution in PQ and control groups. After the membrane protein extraction reagent containing phenylmethanesulfonyl fuluride (PMSF) was added to the cells, the samples were gently and fully suspended and were then placed in the ice bath for 15 minutes. The cells were broken by repeated freezing–thawing 4 times in liquid nitrogen and at room temperature. Then, the samples were centrifuged at 4°C, 700 g for 10 minutes. The supernatant was kept and centrifuged at 4°C, 14 000 g for 30 minutes. The sediments were quantified and saved for Western blot.

Western Blot

Western blot analyses for the expression of GFAP, Apo E, and β-actin were performed as described previously.[19] Briefly, equal amounts of protein were resolved by 10% (vol/vol) sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by electrophoretic transfer to polyvinylidene fluoride (PVDF) membranes. Blots were blocked in Tween-20 Tris-buffered saline containing 3% (wt/vol) nonfat milk for 1 hour at room temperature. Membranes were incubated over night at 4°C with an appropriately diluted primary antibody and were

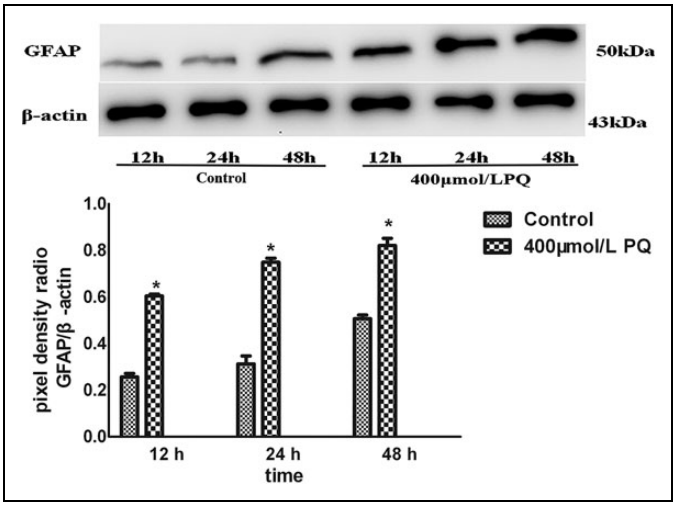

Figure 1. PQ-induced astrocytic reactivity. Protein expression of GFAP increased significantly in the astrocytes exposed to PQ for different time. *Compared to control, $P < .05$. GFAP indicates glial fibrillary acidic protein; PQ, paraquat

then incubated at room temperature with an appropriately diluted secondary antibody for 1 hour with agitation. Visualization of the proteins was performed with an enhanced chemiluminescence system (Amersham Biosciences, Stockholm, Sweden) according to manufacturer's protocol. Density of bands was quantified by densitometry. β-actin was used to normalize the samples.

Statistical Analysis

The data were expressed as mean (SD), and statistical analyses were performed using analysis of variance (ANOVA) and Student *t* test. A *P* value <.05 was considered statistically significant.

Results

Paraquat-Induced Astrocytic Reactivity

Glial fibrillary acidic protein was present in normal astrocytes. After astrocytes were treated with PQ, GFAP expression increased dramatically in the astrocytes (Figure 1), indicating that PQ can induce astrocytic reactivity and cause astrogliosis.

Paraquat Decreased Cholesterol Concentration and ApoE Expression in the Membrane

A noticeable decrease in membrane cholesterol levels (Figure 2) and ApoE expression (Figure 3) were discerned in the astrocytes after being treated with PQ, compared to the levels of control astrocytes. Furthermore, a slight increase in ApoE level was observed in the astrocytes treated with PQ for 48 hours, compared to that treated for 12 hours and 24 hours treatment, but ANOVA showed that there was no statistically significant difference among them.

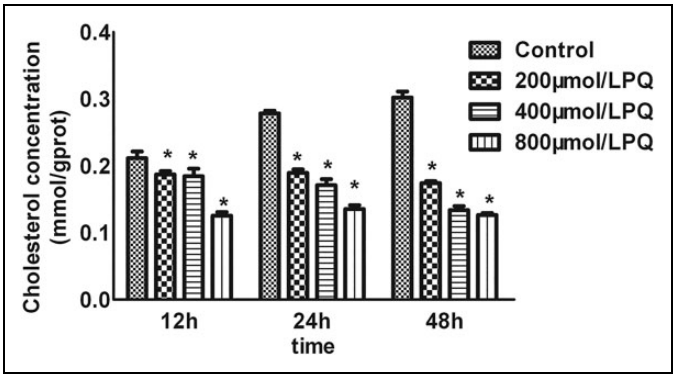

Figure 2. PQ decreased the concentration of cholesterol in the membrane of astrocytes. *Compared to control, $P < .05$. PQ indicates paraquat.

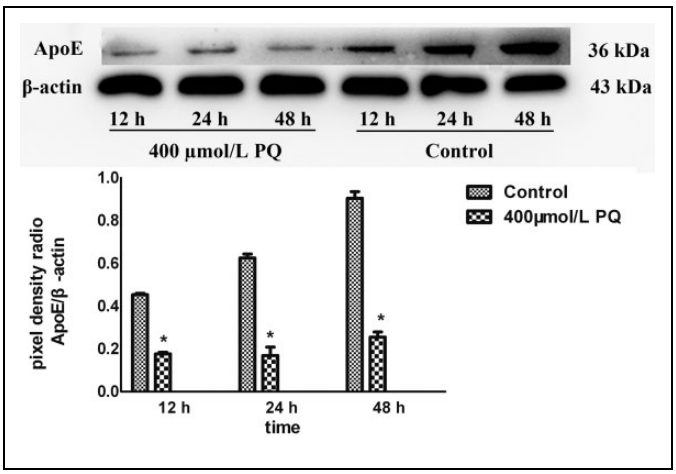

Figure 3. PQ inhibited ApoE expression in the membrane of astrocytes. Protein expression level of ApoE in the membrane of astrocytes decreased significantly after PQ treatment. *Compared to control, $P < .05$. ApoE indicates apolipoprotein E; PQ, paraquat.

Paraquat Altered the Concentration of Glutamate and ATP in the Astrocytes

As shown in Figure 4, the decreasing concentrations of glutamate and ATP were observed in the astrocytes with increasing PQ dosages ($P < .05$), indicating PQ exposure may cause the abnormal astrocytic physiology.

Discussion

Except for providing a passive structural support for neurons, astrocytes actively participate in synaptic transmission and neural communication in the central nervous system (CNS).[12,13] Based on the concept of tripartite synapses, astrocytes integrate and process synaptic information, control synaptic transmission and plasticity, and act as active partners in synaptic function.[20,21] Astrocytes also undergo dynamic structural remodeling in response to physiological or pathological changes in synaptic activity in the CNS.[22] In addition, astrocytes serve as a bridge of nonsynaptic communication

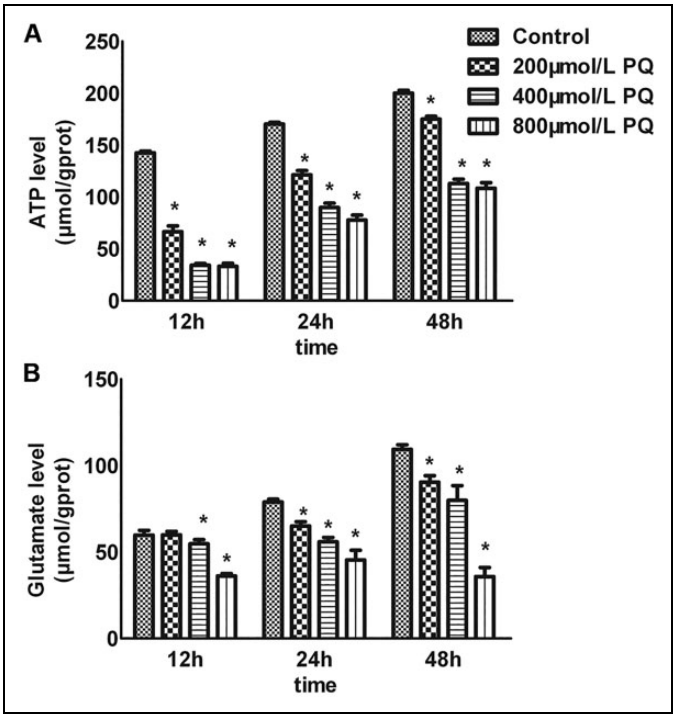

Figure 4. PQ affected the concentration of astrocytic glutamate and ATP. The concentration of ATP (A) and glutamate (B) in the astrocytes decreased after PQ treatment. *Compared to control, $P < .05$. ATP indicates adenosine triphosphate; PQ, paraquat.

between neurons. Therefore, astrocytes not only influence the active synapses through short-range signaling but also have long-range effects on distant synapses.

Astrogliosis is an early pathological feature common to most neurodegenerative diseases and brain injury.[16,23] Reactive astrocytes may be stimulated by specific signaling cascades to gain detrimental effects, such as generation and release of inflammation and ROS.[24,25] Glial fibrillary acidic protein is highly and exclusively expressed in astrocytes. Glial fibrillary acidic protein induction is critically important for the formation of extended and thickened astrocytic processes observed in reactive astrogliosis. Increased GFAP reactivity is considered as a marker of astrogliosis.[26] Here we found that PQ significantly increased GFAP reactivity in astrocytes in vitro, indicating that PQ may contribute to the activation of astrocytes and astrogliosis as observed in brain injury and neurodegenerative diseases. The evidences demonstrate that reactive astrogliosis may promote neural toxicity, damaging nearby neurons via the generation of cytotoxic molecules, such as nitric oxide radicals and other ROS.[27]

Cholesterol released from astrocytes is believed to promote synaptogenesis, and synapse loss is a known by-product of neurodegenerative disease.[28,29] Animal studies demonstrate that neurons rely on external cholesterol supply, and this cholesterol supplement could reasonably derive from glial cells, especially and mainly from astrocytes.[30,31] Also, astrocytes are the main sources of ApoE, a major apolipoprotein in the brain. It is worth mentioning that astrocytic cholesterol is secreted together with ApoE in ApoE-dependent way and then is taken up and utilized by the neurons. So glial cells, including astrocytes, are shown to generate cholesterol-rich and ApoE-containing high-density lipoproteins. Apolipoprotein E synthesis is increased in astrocytes after brain injury, and ApoE is secreted from astrocytes and redistributed to neurons in Parkinson disease.[32,33] The studies from Bajo-Grañeras et al[34,35] demonstrate that apolipoprotein D (ApoD) is necessary for a proper response of the nervous system against physiological and pathological oxidative stress. Apolipoprotein D is a maintenance factor for astrocytes and decreases their reactivity level, which protects astrocytes from oxidative-vulnerable dopaminergic circuits. Previous studies also revealed that deficient ApoE affected the release of cholesterol from astrocytes and was implicated in neurodegenerative diseases.[36,37] In this study, we have found the reduction in cholesterol concentration and decrease in ApoE expression in the membrane of astrocytes after treatment with PQ, suggesting that PQ exposure may be involved in neurodegenerative diseases via altering the release of cholesterol in astrocytes. The evidence has shown that ATP-binding cassette protein A1 (ABCA1) mediates cholesterol efflux and facilitates ApoE secretion as the major transporter from astrocytes. Diminishing activity of the sterol regulatory element-binding protein (SREBP) in astrocytes results in decreased secretion of cholesterol and phospholipid by astrocytes.[31,38] Whether ABCA1 and SREBP expression in astrocytes is affected after PQ exposure needs to be clarified in our future studies.

Astrocytes secrete several neuroactive substances, such as glutamate, ATP, D-serine, TNF-α, and prostaglandins, with different potential neuromodulation of synaptic transmission and neuronal physiology. Compelling evidence shows that these molecules are released in various ways, such as in Ca^{2+}-dependent manner through vesicle and lysosome exocytosis.[21,39-41] Glutamate is one of first identified gliotransmitters released from astrocytes and exerts many effects on neuronal excitability depending on the sites of action and the activated receptor subtypes. For example, astrocytic glutamate evokes slow inward currents through activation of postsynaptic *N*-methyl-D-aspartate receptors (NMDAR) or enhances the frequency of spontaneous and evoked excitatory synaptic currents through activation of metabotropic glutamate receptors or NMDAR at presynaptic terminals.[41-43] Adenosine triphosphate also controls synaptic transmission, and ATP released into synaptic cleft from astrocytes is hydrolyzed by ecto-nucleotidases to yield adenosine which selectively acts on neuronal adenosine receptors to bolster glutamate release and NMDAR to assist increasing synaptic physiology in the activated synapse.[44,45] In present study, the changes in glutamate and ATP concentration in the astrocytes after PQ treatment raise the possibility that the abnormal level of astrocytic glutamate and ATP instigated by PQ might influence their release and further affect the synaptic transmission and astrocytic physiology.

Some neurotrophic factors secreted by astrocytes, such as glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), are potent survival factors for

various neuronal populations. In normal and pathological conditions, GDNF and BDNF exert significant influences on the growth, development, and neuroprotection of dopaminergic neurons and their axonal projections. Our earlier study showed that PQ downregulated the production of GDNF and BDNF by astrocytes in dose- and time-dependent manner, implying that PQ treatment weakened the neuroprotection of astrocytes on dopaminergic neurons.[46]

Conclusion

The present findings add to the growing evidence that PQ may promote neural toxicity by inducing reactive astrogliosis and exerting the detrimental effects on synaptic function and transmission of astrocytes. The changes in astrocytes-regulating synaptic functions caused by PQ may be involved in the progressive nature of the pathophysiology of neurodegenerative disorders. Future studies on the mechanisms involved in the role of astrocytes in neurodegenerative disease will help better understand these processes.

Authors' Note

Zheng Li and Jing Zheng contributed equally to this work.

Declaration of Conflicting Interests

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