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In vitro protective effects of pyrroloquinoline quinone on methylmercury-induced neurotoxicity

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ABSTRACT

Methylmercury (MeHg), as a well-known neurotoxicant, has been implicated to induce massive neurodegeneration. Pyrroloquinoline quinone (PQQ) is a novel redox cofactor and also exists in various plants and animal tissues. In vivo as well as in vitro experimental studies have shown that PQQ functions as an essential nutrient or antioxidant. In this study, we demonstrated the protective effects of PQQ on MeHg-induced neurotoxicity in PC12 cells. The results showed that after pretreatment of PC12 cells with PQQ prior to MeHg exposure, the MeHg-induced cytotoxicity was significantly attenuated, and then DNA fragmentation was correspondingly reduced. PQQ prevented the disruption of mitochondrial membrane potential, up-regulated the level of Bcl-2, and consequently inhibited the activation of caspase-3. Moreover, PQQ also decreased the production of ROS and maintained the GSH levels in PC12 cells exposed to MeHg. Thus, these data indicate that PQQ can protect neurons against MeHg-induced apoptosis and oxidative stress via ameliorating the mitochondrial dysfunction. Data from this study provide a new useful strategy for the treatment of neuronal toxicity induced by mercury toxins.

1. Introduction

Mercury is a ubiquitous environmental contaminant. Methylmercury (MeHg), one organic form of mercury, can easily cross the blood–brain and placental barriers and cause central nervous system (CNS) damage in both the adult and developing brain (Clarkson, 1997; Lapham et al., 1995). Over the last decade, extensive research has been conducted to elucidate the cellular events associated with MeHg-induced neurotoxicity. Investigators have presented work on induction of apoptosis by MeHg in multiple cell types in vitro (Kunimoto, 1994). On the other hand, previous studies on the mechanism of MeHg neurotoxicity have implicated the generation of reactive oxygen species (ROS) and depletion of intracellular glutathione (GSH) as important contributors to observed MeHg-induced cytotoxicity (Sanfeliu et al., 2001). Depletion of GSH associated with MeHg exposure may reduce the cellular ability to destroy free radicals and ROS, so that it eventually triggered the apoptotic cell death.

Pyrroloquinoline quinone (PQQ), a noncovalently bound redox cofactor of bacterial dehydrogenases, was initially isolated from cultures of methylotropic bacteria (Salisbury et al., 1979). As designated in earlier literature, this bacterially synthesized quinone is highly soluble, heat-stable and capable of continuous redox cycling. Following the discovery of PQQ, it has been identified in various fruits, vegetables, milk and even tissues of mammalian animal at pico- or nano-molar levels (Kumazawa et al., 1992, 1995). As an essential nutrient or antioxidant, PQQ has been drawing attention from both the nutritional and the pharmacological viewpoint. Mice fed chemically defined diets devoid of PQQ that are otherwise nutritionally adequate have impaired neonatal growth and abnormal features, including friable skin, evidence of hemorrhage and diverticuli, reduction in general fitness, and a hunched posture (Steinberg et al., 1994). Decreased fertility and defects in immune function also occurs with PQQ deficiency (Steinberg et al., 1994). Recently, it has been proposed that PQQ can be classified as a new B vitamin (Kasahara and Kato, 2003). Moreover, it has been reported that PQQ is a potent neuroprotective nutrient against 6-hydroxydopamine-induced neurotoxicity (Hara et al., 2007).

In epidemiological studies, the general population is exposed to MeHg primarily through ingestion from a diet. Because animal and human body systems do not appear to synthesize PQQ, the diet is also assumed to be the major source (Smidt et al., 1991).

Abbreviations: PQQ, pyrroloquinoline quinone; MeHg, methylmercury; CNS, central nervous system; LDH, lactate dehydrogenase; TUNEL, Tdt-mediated dUTP nick end labeling; Rh123r, hodamine 123; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; DCFH-DA, 2,7-dichlorofluorescein diacetate; DCF, dichlorofluorscein; GSH, glutathione; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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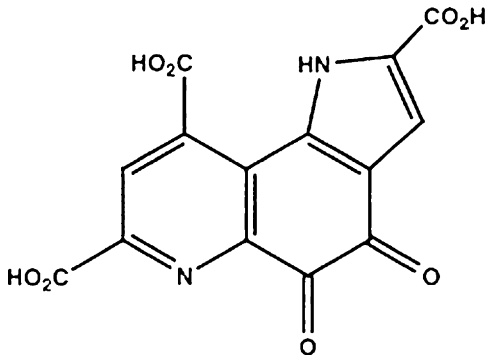

Fig. 1. The chemical structure of pyrroloquinoline quinone (PQQ), shown here as 4,5-dioxo-H-pyrrolo(2,3-*f*) quinoline-2,7,9-tricarboxylic acid.

However, little is known about the potential protective effect of PQQ against MeHg neurotoxicity. Due to its inherent properties mentioned above, it is possible that PQQ protects neurons from MeHg-induced neurotoxicity.

Dopamine neurons are implicated in a wide variety of functions such as attention, cognition, motor, and reward-related behaviours. Moreover, a number of neurological pathologies, including Parkinson's disease, schizophrenia, mood disorders, as well as deficits in attention, motor control, and perception have been associated to dysfunctions of the dopaminergic system. The toxic effects of MeHg on the developing dopaminergic system might predispose to the onset of pathological conditions later in life (Cernichiari et al., 1995). PC12 cell line was established from rat adrenal pheochromocytoma cell (Greene and Tischler, 1976). The membrane receptors and synthesized transmitters in PC12 cells are similar to dopaminergic neurons located in midbrain. The current investigation capitalized on the fact that undifferentiated PC12 cells represents immature neurons that are most vulnerable to the effects of MeHg (Igata, 1993; Harada, 1995). Therefore, we focused on the effects of PQQ on MeHg-induced neurotoxicity in undifferentiated PC12 cells in order to provide experimental basis for the treatment of neuronal toxicity induced by mercury toxins.

2. Materials and methods

2.1. Reagents

Pyrroloquinoline quinone (PQQ, Fig. 1) was obtained from Shanghai Medical Life Science (Shanghai, China). Methylmercury chloride (MeHg) and 2,7-dichlorofluorescein diacetate (DCFH-DA) and Rhodamine 123 (Rh123) were purchased from Sigma Chemical (St. Louis, MO, USA). RPMI 1640 and equine serum were purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Company (Hangzhou, China). The monoclonal antibodies to Bcl-2 and Bax were purchased from Beijing Zhongshan Biotechnology Co. Ltd. (Beijing, China). The fluorescein isocyanate (FITC)-conjugated secondary antibody was obtained from Sigma Chemical (St. Louis, MO, USA). Lactate dehydrogenase (LDH) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). GSH assay kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). Caspase-3 activity kit was purchased from Nanjing Keygen Biotech. Co. Ltd. (Nanjing, China).

2.2. Cell culture

PC12 cells were obtained from Peking Union Medical College (Beijing, China) and cultured in RPMI 1640 medium supplemented with 10% (v/v) equine serum and 5% (v/v) fetal bovine serum. Cells were maintained at 37 °C in an air and 5% CO_2 atmosphere.

2.3. Cytotoxicity assay

Cytotoxicity was quantitatively assessed by measuring the activity of LDH released from the damaged cells into the culture medium (Vian et al., 1995). Briefly, PC12 cells were plated at density of 8.0×10^5 cells per well in 6-well plates. After 24 h, cells were exposed to various concentrations of MeHg (2, 4, 6, 8 and 10 μM) for 4 h, or pretreated with different concentrations of PQQ (3, 30, 300, 3000 and 30,000 nM) for 30 min, and then were exposed to 6 μM MeHg for 4 h. To test the toxicity of PQQ to PC12 cells, we also treated cells with PQQ for 4 h. At the end of treatments, the cell suspension was centrifuged at 4000 × *g* at 4 °C for 5 min, and then the supernatants were collected, whereas the cell pellets were lysed with cell lysis buffer containing 1% Trition X-100. LDH assays in supernatant aliquots and lysates were performed by using the cytotoxicity assay kit according to the manufacturer's protocol. The wavelength to measure absorbance was 440 nm and LDH expressed cytotoxicity (%) was calculated using the formula: (supernatant value − blank value)/[(supernatant value − blank value) + (lysates value − blank value)] × 100%.

2.4. Quantification of DNA fragmentation in apoptosis

DNA fragmentation was determined by using the Tdt-mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer's protocol. Briefly, PC12 cells were plated in 6-well plates (8×10^5 per well). After 24 h, cells were treated with different concentrations of MeHg (2, 4, 6 and 8 μM) for 4 h, or pretreated with PQQ (3, 30 and 300 nM) for 30 min, and then were exposed to 6 μM MeHg for 4 h. Cells were washed with cold PBS, fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 on ice for 2 min, and then incubated with 50 μl TUNEL

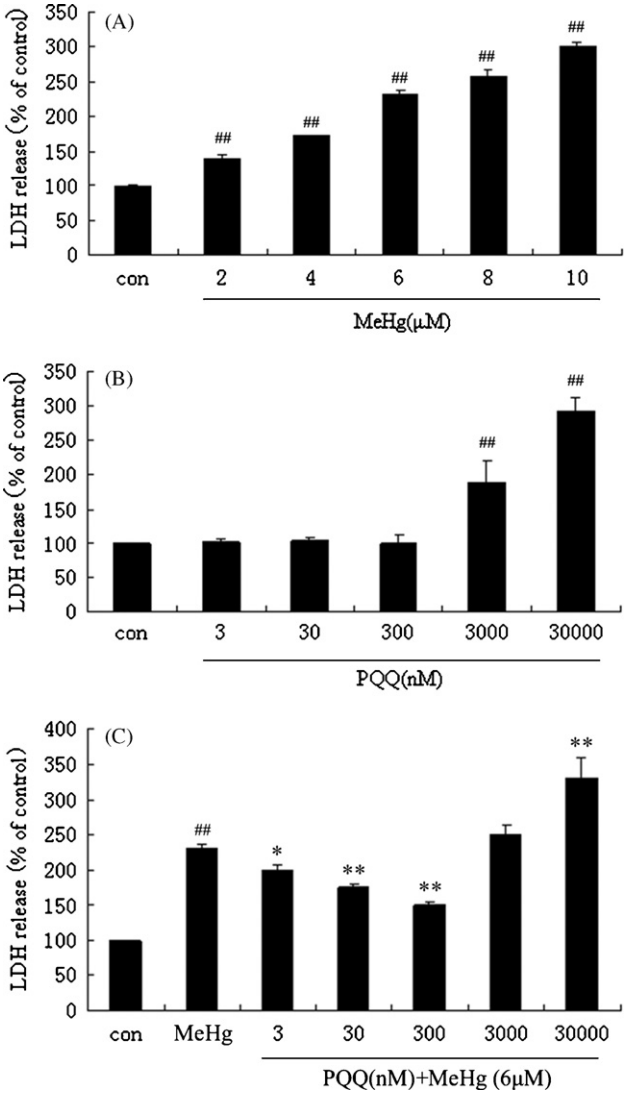

Fig. 2. (A) Effect of MeHg on LDH release in PC12 cells. PC12 cells were treated with MeHg for 4 h. (B) Effect of PQQ on LDH release in PC12 cells. PC12 cells were treated with PQQ for 4 h. (C) Effect of PQQ on LDH release in PC12 cells exposed to MeHg. PC12 cells were pretreated with PQQ for 30 min, and then were exposed to 6 μM of MeHg for 4 h. After the treatment, LDH release in cell suspensions and the total LDH were measured. Values are means ± S.D. of triplicate independently experiments. $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, compared with control, $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with 6 μM MeHg treatment group.