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Effect of GADD45a on olaquindox-induced apoptosis in human hepatoma G2 cells: Involvement of mitochondrial dysfunction

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ABSTRACT

Olaquindox, a quinoxaline 1, 4-dioxide derivative, has been widely used as a feed additive for promoting animal growth in China. The aim of present study was to investigate the effect of grow arrest and DNA damage 45 alpha (GADD45a) on olaquindox-induced apoptosis in HepG2 cells. The result showed that olaquindox induced the decrease of cell viability in a dose dependent manner. Compared to the control group, olaquindox treatment at 400 and 800 μg/mL increased the expression level of GADD45a protein and reactive oxygen species (ROS) production, decreased mitochondrial membrane potential (MMP), and subsequently increased the expression of Bax while decreased the expression of Bcl-2, leading to the release of cytochrome c (Cyt c). However, knockdown of GADD45a enhanced olaquindox-induced ROS production, disrupted MMP and subsequently caused Cyt c release, then further increased olaquindox- induced cell apoptosis by increasing the activities of caspase-9, caspase-3, and poly (ADP-ribose) polymerase (PARP). In conclusion, the results revealed that GADD45a played a critical role in olaquindox-induced apoptosis in HepG2 cells, which may embrace the regulatory ability on the mitochondrial apoptosis pathway.

1. Introduction

Quinoxaline 1, 4-dioxides (QdNOs) is a class of quinoxaline derivatives consisting of one or two acyclic chains moiety combined with quinoxaline ring (Cheng et al., 2015). It has been shown that QdNOs possess many biological activities, such as antibacterial, anticancer and antiprotozoal capabilities. Olaquindox is a member of quinoxaline 1, 4-dioxide (QdNOs) family and it has been widely used as an animal feed additive to promote animal growth and improve feed conversion rate in China (Zhao et al., 2015). Recent literature showed that olaquindox could cause the potential genotoxicity *in vitro* and *in vivo* (Ihsan et al., 2013). Up to now, due to lack of the evidence of genotoxicity caused by olaquindox in animal production and its unclear mechanism, olaquindox is still been largely used as the most common animal feed additive in pig production in China (Yang et al., 2010). Commission of the European community and Health Canada have prohibited the use of olaquindox as an animal growth promoter in 1999.

A long-term toxicity study of olaquindox in rats showed that the toxic effects were observed in the liver, kidney, testes, ovaries and endocrine glands (Fang et al., 2006). Olaquindox has been regarded as the strong mutagen in both TA98 and TA100 strains of Salmonella typhimurium (Yoshimura et al., 1981). Moreover, olaquindox was cytotoxic and genotoxic to Vero and HepG2 cells (Chen et al., 2009). Treatment with olaquindox produced serious chromosome damage and DNA damage in HepG2 cells (Zou et al., 2009). ROS production and GSH depletion were considered as the major mechanism of olaquindox-induced apoptosis. In our previous study, olaquindox induced apoptosis may relate with p38 MAPK and ROS-mediated JNK pathways in HepG2 cells (Zhao et al., 2013). Zou showed that olaquindox arrested cell cycle and induced apoptosis in a dose-dependent manner in HepG2 cells, suggesting that apoptosis is a major mechanism of olaquindox-induced cell death (Zou et al., 2011).

The growth arrest and DNA damage 45 alpha (GADD45a) have been implicated in regulation of many cellular functions including DNA repair, cell cycle control, senescence and genotoxic stress (Zhan, 2005). GADD45a is known to be regulated by both p53-dependent and -independent pathways depending on the type of genotoxic stress (Siafakas and Richardson, 2009). It has been

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showed that GADD45a was regulated by p53-dependent pathway treated with IR (Zhan et al., 1994) and p53-independent pathway treated with UV (Hildesheim et al., 2002). In an earlier study, GADD45a played a pro-apoptotic role during isopropyl beta-D-thiogalactopyranoside (IPTG) induced DNA injury in M1 cell and H1299 (Zhang et al., 2001). On the contrary, some findings suggested that GADD45a is an anti-apoptotic gene enhancing the survival of cells treated with anticancer drugs, such as VP-16 and daunorubicin (DNR) (Gupta et al., 2006). Recent studies indicated that GADD45a functions as either tumor suppressor or promoter is dependent on stimulation of oncogenic stress (Tront et al., 2010). Our previous study showed that olaquindox treated HepG2 cells up-regulated p53, induced cell apoptosis and cell cycle arrest (Chen et al., 2009; Zou et al., 2011). GADD45a may play a critical role in olaquindox-induced cell death. In present study, the cytotoxic effect of olaquindox in HepG2 cells was investigated and the underlying mechanism was further discussed.

2. Material and method

2.1. Material

Olaquindox (CAS NO.23696-28-8, purity ≥98%) was purchased from China Institute of Veterinary Drug Control (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies Corporation (Grand Island, NY, USA). Dimethyl Sulphoxide (DMSO), 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) were all obtained from AMRESCO (Solon, OH, USA). Sodium pyruvate and PMSF were all obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of reagent grade.

2.2. Cell culture and treatment

HepG2 cell was purchased from American Type Culture Collection (ATCC). Cells cultured in Dulbecco minimal Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) containing 2% L-glutamine, 10% fetal bovine serum (Gibco, USA), 1% penicillin and streptomycin (Beyotime Institute of Biotechnology Co., Ltd., Haimen, China) at 37 °C in 5% CO_2. Cells were treated with Olaquindox at the final concentration of 200, 400, 800 μg/mL, respectively. In the control group, cells were treated with 0.1% DMSO.

2.3. Target gene GADD45a-RNAi lentiviral packaging

GADD45a interference sequence designed by Ambion's software which based on target gene was adopted. The shRNA target sequence of GADD45a is 5′-CCT GCC TTA AGT CAA CTT ATT-3′ and the shRNA sequences of control is 5′-TGA CAT GAT AAT ACT CTC T-3′, without interference suppression on the expression of human gene. HepG2 cells were transfected with the pLKO.1 plasmid containing either control shRNA (HepG2) or GADD45a specific shRNA (HepG2-iGADD45a), using X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland). Twenty-four hours after transfection, cells were re-suspended, and exposed to puromycin (1 mg/mL). Transfection efficiency was determined 14 days after transfection.

2.4. Measurement of cell viability

The cell viability was examined using MTT method. Cells (1×10^4 cells per well) were seeded in 96-well culture plates the day before olaquindox treatment. After treatment, the medium which containing olaquindox was removed, and cells were incubated in the 100 μL fresh medium supplemented with 10 μL MTT solution (5 mg/mL) for 4 h at 37 °C. At the end of incubation, the medium was gentle removed and 100 μL DMSO was added into each well. The optical density was assessed at 570 nm in a micro plate reader (Molecular Devices, Sunnyvale, CA, USA). Cell survival was expressed as percentage of the control.

2.5. Measurement of apoptosis

Cells were planted into 6-well plate. After 24 h, cells were treated with olaquindox (0, 200, 400 and 800 μg/mL) at 37 °C in a humidified 5% CO_2 for 24 h. Flow cytometric analysis of apoptosis in cells were carried out using an annexin V-FITC apoptosis detection kit (Vazyme Biotech Co., Ltd., Nanjing, China). Cells were detached with 0.25% trypsin without EDTA, washed twice with phosphate buffer saline (PBS) and re-suspended in 500 μL binding buffer. Following that, cells were incubated with 5 μL annexin V-FITC and 5 μL propidium iodide for 15 min at room temperature in a dark environment and analyzed by BD FACSAria™ flow cytometer using BD FACSDiva Software (BD Biosciences, CA, USA) within 1 h.

For Hoechst 33342 staining, cells (1×10^5 cells per well) were cultured on 6-well culture plates and treated with olaquindox (0, 200, 400 and 800 μg/mL) at 37 °C for 24 h. After staining in the dark with 1 μg/mL Hoechst 33342 (Vigorous Biotechnology, Beijing, China) for 20 min, the cells were observed under a fluorescence microscope (excitation wavelength at 340 nm and emission wavelength at 510 nm) (Leica Microsystems, Wetzlar, Germany). Cells which showed chromatin condensation or DNA fragmentation that were counted as apoptotic cells.

2.6. Western blotting analysis

Cells were collected and lysed in a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 2% SDS, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4 and 10 μl PMSF. An equal amount of cellular protein was loaded into each well of sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins were transferred to nitrocellulose membranes (Mini-Protean and Trans-Blot systems, Bio-Rad Laboratories, Hercules, CA, USA). Themembranes were blocked with milk and were incubated with specific primary and secondary antibodies. The blots were detected using western luminescent detection kit (Vigorous Biotechnology, Beijing, China). The following primary antibodies were employed: rabbit polyclonal antibodies against caspase3 (1:500), caspase9 (1:500) (Zhongshan Golden Bridge Co., Beijing, China), PARP (1:1000; Beyotime Institute of Biotechnology Co., Ltd. Haimen, Jiangsu, China). Bax (1:3000), Bcl-2 (1:1000) (Protein-Tech Group, Inc., Chicago, IL, USA); mouse monoclonal antibody against GADD45a (1:500; Santa Cruz, CA, USA), Cyt c (1:2000), GAPDH (1:1000), and β-actin (1:1000; Zhongshan Golden Bridge Co., Beijing, China). The secondary antibodies employed were goat anti-rabbit IgG (1:5000) or rabbit anti-mouse IgG (1:5000) (Zhongshan Golden Bridge Co., Beijing, China).

2.7. Measurement of reactive oxygen species production

The intracellular ROS was measured with cell-permeant probe DCFH-DA. Briefly, cells were treated with olaquindox at the final concentrations of 0, 200, 400 and 800 μg/mL for 24 h. Cells were washed with phosphate-buffered saline and incubated with 10 μM DCFH-DA at 37 °C for 30 min. The cells were then harvested and analyzed by the flow cytometer.

2.8. Measurement of mitochondrial membrane potential (MMP)

The alteration of cellular MMP from olaquindox exposure was determined by Rhodamin 123 (Sigma Chemical Co., MO, USA). Cells were plated into 6 well plates and treated with the concentration