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Original article

Psoralidin induced reactive oxygen species (ROS)-dependent DNA damage and protective autophagy mediated by NOX4 in breast cancer cells

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ABSTRACT

Background: Psoralidin (PSO), a natural phenolic coumarin, was reported to have anti-cancer activities. PSO induced reactive oxygen species (ROS) generation in cancer cells. The role of ROS in its anti-cancer effect remains unclear.
Purpose: This study was designed to investigate the potential roles of ROS in PSO-induced anti-cancer effect in MCF-7 breast cancer cells.
Methods: Effect of PSO on cancer cell proliferation was determined by MTT assay. Comet assay was used to determine DNA damage. Protein expression was detected by Western blotting. Autophagic vacuoles were detected by monodansylcadaverine (MDC) staining. ROS generation was measured by fluorescent probe. NOX4 localization was determined by immunofluorescence staining.
Results: PSO treatment caused proliferation inhibition in time- and dose- dependent manners, which was partially reversed by N-acetyl cysteine (NAC) and diphenyleneiodonium (DPI). PSO induced DNA damage and increased protein expression of γ-H_2AX, phosphorylation of ATM, ATR, Chk1, and Chk2. PSO induced autophagy as evidenced by the accumulation of autophagic vacuoles and alterations of autophagic protein expression. PSO-induced cell death was enhanced by autophagy inhibitor chloroquine (CQ). Furthermore, PSO treatment induced ROS formation, which was reversed by NAC or DPI pretreatment. The expression of NOX4 was significantly enhanced by PSO. Both NAC and DPI could reverse PSO-induced DNA damage and autophagic responses. In addition, silencing NOX4 by siRNA inhibited PSO-induced ROS generation, DNA damage, and autophagy.
Conclusions: Taken together, these results showed that PSO induced DNA damage and protective autophagy mediated by ROS generation in a NOX4-dependent manner in MCF-7 cells.

Introduction

Psoralea corylifolia L. (Buguzhi) (*P. corylifolia*) is an important medical plant used in Ayurvedic medicine as well as in traditional Chinese medicine (TCM). In TCM, this herb has been used for several centuries with the traditional function of reinforcing and tonifying the kidney yang. Modern researches reported that the plant extracts possessed antibacterial, antitumor, antioxidant, anti-inflammatory, antifungal, and immunomodulatory activities (Chopra et al. 2013). Phytochemical investigations have identified approximate 90 compounds including psoralen, isopsoralen, psoralenoside, isopsoralenoside, bakuchiol, psoralidin (PSO), bakuchicin, bakuchalcone, bavachinin, flavonoids, and meroterpenes *etc* from the seeds or fruits of *P. corylifolia* (Zhang et al. 2016). PSO is a furanocoumarin firstly isolated from the seeds of *P. corylifolia* in 1948. Its chemical structure was identified in 1961 and was totally synthesized in 2009 (Pahari and Rohr 2009). Documented data demonstrated that PSO possessed antibacterial (Khatune et al. 2004), radio-preventive (Lobrich et al. 2010), and anti-inflammatory effects (Chiou et al. 2011; Matsuda et al. 2009). Accumulated evidence suggested that PSO possessed anticancer potentials. PSO inhibited the proliferation of a number of cancer cells in vitro such as the stomach carcinoma line cells SNU-1 and

Abbreviations: ATCC, American type culture collection; CQ, Chloroquine; $DCFH_2$-DA, 5-(6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; DPI, diphenyleneiodonium chloride; MDC, monodansylcadaverine; MTT, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NAC, N-acetyl cysteine; PI, propidium iodide; PSO, Psoralidin; ROS, Reactive oxygen species; TCM, Traditional Chinese medicine.

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SNU-16 (Yang et al. 1996), the human colon cancer HT-29 cells and the breast cancer MCF-7 cells (Mar et al. 2001), the prostate cancer PC-3 cells (Das et al. 2014), and the human lung cancer A549 cells (Hao et al. 2014). Furthermore, PSO enhanced TRAIL-mediated apoptosis in prostate cancer cells (Szliszka et al. 2011) and HeLa cells (Bronikowska et al. 2012). Recent study also indicated that PSO-induced reactive oxygen species (ROS) generation inhibited epithelial-mesenchymal transition (EMT) and promoted growth arrest in prostate cancer cells (Das et al. 2014). More recently, PSO was identified as a full estrogen receptor (ER) agonist, activating the classical ER-signaling pathway and inducing the endogenous estrogen-responsive gene pS2 in MCF-7 cells (Liu et al. 2014). However, the detailed mechanisms of its anticancer activities remain unclear.

Oxidative stress resulted from excessive generation of ROS from mitochondria, NADPH oxidase *etc* induces multiple biological responses, such as DNA damage, cell cycle arrest, autophagy, and apoptosis. Our previous study demonstrated that PSO inhibited the proliferation of A549 cells mediated by autophagy through ROS generation (Hao et al. 2014). Furthermore, PSO-induced ROS played an important role in inhibiting EMT and promoting growth arrest in prostate cancer cells (Das et al. 2014). However, the detailed roles of ROS in PSO-induced cell death remain unclear. NADPH oxidase (NOXs), one of the main ROS sources in biological systems, plays important roles in cancer chemotherapy. There are 7 members of NOXs including NOXs 1–5 and dual oxidases DUOX1/2 (Meitzler et al. 2014; Roy et al. 2015). Among these members, recent attention has been paid to the role of NOX4 in cancer (Guo and Chen 2015). In this study, the potential role of NOX4 in PSO-induced cell death was investigated in MCF-7 cells.

Materials and methods

Reagents

PSO (> 98%) was purchased from Chengdu Preferred Biotech Co. Ltd. (Chengdu, China). 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), monodansylcadaverine (MDC), DAPI, N-acetyl cysteine (NAC), 5-(6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), diphenyleneiodonium chloride (DPI), and chloroquine (CQ) were obtained from Sigma Aldrich (St. Louis, MO, USA). Antibodies against γ-H_2AX, ATM, p-ATM (Ser1981), ATR, p-ATR (Ser428), Chk1, p-Chk1 (Ser345), Chk2, p-Chk2 (Thr68), AKT, p-AKT (Ser473), mTOR, p-mTOR (Ser2448), ULK1, p-ULK1 (Ser317), Beclin-1, p62, LC3, NOX1, NOX2, NOX4, NOX5, p22, Rac1, and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Adenocarcinomic human alveolar basal epithelial A549 cells, human breast cancer MCF-7 cells, and MDA-MB-231 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). MCF-7 and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium and A549 cells were maintained in RPMI 1640 Medium, supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin in a 5% CO_2 atmosphere at 37 °C.

MTT assay

Exponentially growing cells were seeded in 96-well plates and were added MTT mixtures (5 mg/ml) after PSO (2.5–10 μM) treatment. The formazan was solubilized by DMSO to detected by the Multilabel counter after incubated for another 4 h. Cells were pre-treated with NAC (5 mM), DPI (10 μM), or CQ (10 μM) for 1 h before PSO treatment.

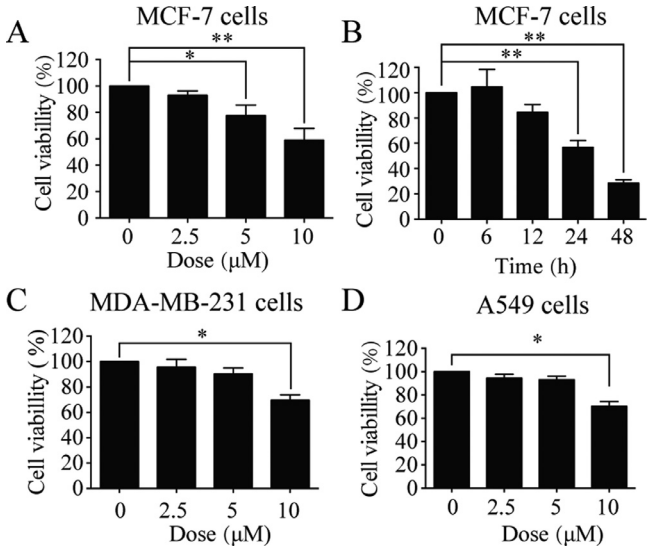

Fig. 1. The anti-proliferative effects of PSO on cancer cells. (A). MCF-7 cells were treated with PSO (0–10 μM) for 24 h and the cell viability was determined by MTT assay. (B). MCF-7 cells were treated with PSO (10 μM) and the cell viability was determined by MTT assay. MDA-MB-231 (C), and A549 (D) cells were treated with PSO (0–10 μM) for 24 h and the cell viability was determined by MTT assay. PSO, psoralidin. $^{**}p < 0.01$,* $p < 0.05$.

Comet assay

After treatment with PSO (10 μM) for 3 h, cells (1×10^5) were harvested and the comet assay was performed as our previous study (Guo et al. 2014). To explore the role of ROS and NADPH oxidase, cells were pretreated with NAC (5 mM) or DPI (10 μM) for 1 h before PSO treatment.

Immunofluorescence assay

Cells (1×10^4) in 96-well plates were fixed with 4% paraformaldehyde at room temperature for 30 min after PSO treatment for 3 h. Then cells were permeabilized with PBS containing 0.2% Triton at room temperature for 30 min and then blocked with blocking buffer containing 2.5% bovine serum albumin (BSA) and 0.2% Triton in PBS for 30 min at 4 °C. Cells were incubated with NOX4 antibody (1:1000) overnight and then washed with PBS twice and incubated with anti-rat IgG (1:2000) at room temperature for another 1 h. Nuclei were stained for 10 min in the dark with DAPI and cells were observed under a fluorescence inverted microscope using filter set for Cy3 and DAPI.

MDC staining

Cells (1×10^4) were seeded 96-well plates and incubated overnight. After treatment with PSO, cells were incubated with MDC (50 μM) in PBS for 30 min and then washed three times with PBS and immediately visualized by a fluorescent microscopy. Cells were pretreated with NAC (5 mM) or DPI (10 μM) for 1 h before PSO (10 μM) treatment.

SiRNA transfection

Briefly, cells seeded in 6-well plates (3×10^5/well) for overnight were added 100 pM siRNA in 100 μl Opti-MEM serum-free medium and mixed gently. Diluted 5 μl lipofectamineTM 3000