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Triptolide induces p53-dependent cardiotoxicity through mitochondrial membrane permeabilization in cardiomyocytes

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ABSTRACT

Triptolide (TP), a major active component of *Tripterygium wilfordii Hook f.*, is widely used in the treatment of inflammation and autoimmune disorders. Its clinical application is limited by severe adverse effects, especially cardiotoxicity. Accumulative evidences indicate that TP induces DNA damage by inhibiting RNA polymerase. Considering the relationship among DNA damage, p53, and the role of p53 in mitochondria-dependent apoptosis, we speculate that TP-induced cardiotoxicity results from p53 activation. In this study, the role of p53 in TP-induced cardiotoxicity was investigated in H9c2 cells, primary cardiomyocytes, and C57BL/6 genetic background $p53^{-/-}$ mice. p53 protein level was elevated by TP *in vitro* and in acute heart injury models. With TP administration (1.2 mg/kg), *p53* deficiency prevented heart histology injury and decreased serum cardiac troponin I (cTn-I) and apoptotic proteins. Mechanistically, immunoblotting and immunofluorescence staining identified that TP-induced toxicity is dependent on p53 nuclear translocation and transactivation of Bcl2 family genes, leading to mitochondrial outer membrane permeabilization (MOMP) and mitochondria dysfunction. Consistently, p53 antagonist PFTα counteracted TP-induced p53 overexpression and regulation of Bcl2 family transcription, which improved mitochondrial membrane integrity and prevented apoptosis. Moreover, Bax antagonist Bax inhibitor peptide (BIP) V5 ameliorated TP-induced apoptosis through suppressing membrane depolarization and ROS accumulation. These results suggest that TP-induced cardiotoxicity is p53-dependent by promoting Bax-induced mitochondria-mediated apoptosis.

1. Introduction

Tripterygium wilfordii Hook f. (TWHF) is a traditional Chinese medicine (TCM) that has been widely used for the treatment of autoimmune and inflammatory diseases for centuries (Shamon et al., 1997; Tu, 2009). The extracts of TWHF have been approved as a therapeutic agent for rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and ankylosing spondylitis (AS) by the China Food and Drug Administration (CFDA) (Ji et al., 2010). Triptolide (TP), a highly promising diterpenoid triepoxide in TWHF (Kupchan et al., 1972), has been reported to exert multiple biological activities including anti-inflammatory, antifertility, antineoplastic, and immunosuppressive effects (Gu and Brandwein, 1998; Huynh et al., 2000; Shamon et al., 1997; Yang et al., 1992). Despite the various pharmacological potencies, the clinical applications of TP are limited by the narrow therapeutic window and severe toxicity in renal, hepatic, reproductive, and cardiovascular systems (Huynh et al., 2000; Li et al., 2012, 2014a, 2014b; Zhou et al., 2014).

Among the adverse effects of TP, TP-induced toxicity in heart tissues is a common occurrence. As reported in clinical cases, the preparations of TWHF elicited chest distress, cardiopalmus, bradyarrhythmia, and even cardiogenic shock, which caused death, and in autopsy cases there were obvious pathological changes in myocardia such as hydropic degeneration and contraction band necrosis (Chou et al., 1995; Huang et al., 2009; Zhou et al., 2014). Our previous studies demonstrated that TP induced cardiomyocyte damage and cell apoptosis *in vitro* and *in vivo* (Wang et al., 2016; Zhou et al., 2014, 2015). However, the underlying mechanism of TP-induced cardiotoxicity has not been adequately investigated.

The p53 tumor-suppressor protein responds to various cellular stress signals such as DNA damage in apoptosis (Brady et al., 2011). p53

Abbreviations: TP, Triptolide; TWHF, *Tripterygium wilfordii Hook f*; MOMP, mitochondrial outer membrane permeanilization; PFTα, Pifithrin-α; BIP V5, Bax inhibitor peptide V5; DOX, Doxorubicin; TUNEL Assay, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling assay; DCFH-DA, 2, 7-dichlorofluorescin diacetate; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; SPF, Specific pathogen free; cTn-I, serum cardiac troponin I; TCM, Traditional Chinese Medicine

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usually localizes in the cytoplasm with a low protein level. In the presence of cellular stresses, p53 is stabilized and increased to induce cell apoptosis (Brooks and Gu, 2011b). In p53 transcription-dependent apoptotic pathway, p53 acts as a transcription factor regulating the transcription of its target genes such as death receptor Fas and Bcl2 family members including Puma, Bcl-2, Bcl-xL, Bax and Bak which lead to the mitochondrial membrane permeabilization (Chiu et al., 2003; Riley et al., 2008; Wolff et al., 2008).

The mitochondrial pathway of apoptosis is the most common mechanism of apoptosis in vertebrates. In this process, mitochondrial membrane permeabilization triggered by stimuli is critical to liberating essential apoptotic factors sequestered in the intermembranous space. These factors assemble apoptosome (Bratton and Salvesen, 2010; Bratton et al., 2001) to activate the specific initiator caspase-9, which ultimately activates caspase proteases and cleaves intracellular proteins, leading to the dismantling of the cell (Green and Llambi, 2015; Tait and Green, 2010). Mitochondrial outer membrane integrity is tightly regulated by Bcl2 protein family (Cory and Adams, 2002). The proapoptotic effector proteins (Bax and Bak) are necessary and sufficient for MOMP. Upon activation, they oligomerize and insert into mitochondrial membrane, form a Bax/Bak-lipid pore, and eventually cause MOMP. The antiapoptotic Bcl2 proteins (Bcl-2, Bcl-xL) block MOMP by binding to Bax/Bak and sequestering them from the BH3-only proteins (Chipuk et al., 2010; Youle and Strasser, 2008).

Accumulative evidences indicate that TP induces DNA damage by binding to the XPB subunit of the general transcription factor TFIIH (Titov et al., 2011), which could activate p53 as an effector protein in DNA repair, cell-cycle arrest, senescence, and apoptosis (Zheng et al., 2017). Accumulating evidence suggests that p53 is pivotal in determining cardiomyocyte death. Herein, we explored the role of p53 protein in the TP-induced cardiomyocyte toxicity *in vitro* and *in vivo*. Indeed, our results demonstrated a critical role of p53 in TP-induced acute heart injury.

2. Materials and methods

2.1. Reagents

TP (> 99% purity) was purchased from Shanghai DND PharmTechnology Co. (Shanghai, China). p53 inhibitor Pifithrin-α (PFTα) (97.21% purity) was purchased from Selleck Chemicals (Houston, TX,USA). Bax inhibitor peptide V5 (BIP V5) (99.79% purity) was purchased from MedChem Express (Mountain Junction, NJ, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich Biotechnology (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from HyClone (GE Healthcare, Chicago, IL, USA). Trypsin were purchased from Sigma-Aldrich Biotechnology (St. Louis, MO, USA). Anti-p53 antibody, anti-Bax antibody, anti-Bak antibody, anti-Bcl-2 antibody, anti-Bcl-xL antibody, anti-Cyt C antibody, anti-Caspase-3 antibody, anti-Caspase-9 antibody, anti-Puma antibody, anti-Fas antibody, anti-COX VI antibody, and anti-β-actin antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse cTn-I ELISA kit was purchased from Shanghai Enzyme Biotechnology (Shanghai, China).Other chemicals were of analytical grade from commercial suppliers.

2.2. Cell cultures

H9c2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and were cultured in DMEM supplemented with 10% FBS. Cells were grown under 5% CO_2 at 37 °C in a humidified incubator. Cell culture materials were purchased from NEST Biotech (Nest Biotechnology, Jiangsu, China).

2.3. Primary culture of rat neonatal cardiomyocytes

The Sprague–Dawley (SD) rats were supplied by the Laboratory Animal Center of Sun Yat-Sen University (Guangzhou, PR China). One- to 3-day-old SD rats were euthanized using ethyl ether before disinfected with 75% ethanol. Hearts were obtained and immediately placed in cold Ca^{2+} and Mg^{2+}-free PBS buffer for washing and mincing. After repetitive preconditioning with trypsin (0.08% trypsin solution) on ice, the minced pieces were digested 10 to 12 times with the same trypsin solution at 37 °C. Cell suspension from each digestion was collected and centrifuged for at least 5 min to exclude the precipitates. The cells were finally harvested by centrifugation for 6 min at 1000*g* and were then plated in a culture flask for 1 h in a humidified incubator including 5% CO_2 at 37 °C to exclude the nonmyocytes. The purified cardiomyocytes were cultured continuously in DMEM containing 10% FBS and 0.1 mM 5-bromodeoxyuridine.

2.4. Cell viability assay

H9c2 cells were seeded in 96-well plates at a density of 4×10^3 cells/well and were used for experiments after 24 h. The cells were treated with control (0.1% DMSO) or PFTα (10 μM) or BIP V5 (100 μM) for 1 h and were exposed with TP solution (160 nM) for 24 h. After cotreatment, 10 μL of 5 mg/mL MTT were directly added to the cells, then incubated for 4 h at 37 °C in incubator. 96-well plates were centrifuged at 1500 rpm for 5 min, then the medium was discarded. Precipitate was solubilized with DMSO (150 μL). After shaking at 37 °C for 10 min, the absorbance of the dissolved formazan grains within the cells was measured at 492 nm using a microplate reader (Thermo Multskan Ascent 354, USA).

2.5. Intracellular ROS accumulation detection

The fluorescent probe 2, 7-dichlorofluorescin diacetate (DCFH-DA) was used to determine the intracellular accumulation of reactive oxygen species (ROS) (Sigma, St. Louis, MO, USA). DCFH-DA, a cell-permeable non-fluorescent probe, is de-esterified intracellularly and forms to highly fluorescent DCFH upon oxidation. Briefly, H9c2 cells were seeded in 24-well plates at a density of 4×10^4 cells/well. Consistent with treatment in the MTT assay as previously described, the cells were incubated with TP (160 nM) for 24 h after pretreatment with PFTα (10 μM) or BIP V5 (100 μM) for 1 h. And then 100 μL DCFH-DA (10 μM) was added to each well incubated at 37 °C for 20 min. After washing three times with PBS, the fluorescence was photographed immediately using High Content Screening (ArrayScanVTI, Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Analysis of apoptosis by TUNEL histology

To evaluate the apoptotic response in heart tissue, we applied terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique to formalin-fixed myocardial samples in paraffin blocks, using the commercially available Colorimetric TUNEL Apoptosis Assay Kits (Roche, Basel, Switzerland). The sections (3 μm) mounted on glass slides were deparaffinized, rehydrated through graded alcohols to water, treated with 20 μg/mL proteinase K (37 °C, 20 min), and then washed three times with PBS. Sections were