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Inhibition of glycogen synthase kinase 3beta ameliorates triptolide-induced acute cardiac injury by desensitizing mitochondrial permeability transition

Wenwen Wang [a], Yanqin Yang [a], Zhewen Xiong [a], Jiamin Kong [a], Xinlu Fu [b], Feihai Shen [a,*], Zhiying Huang [a,*]

[a] School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, PR China
[b] Laboratory Animals Center, Sun Yat-sen University, Guangzhou 510006, PR China

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ABSTRACT

Triptolide (TP), a diterpene triepoxide, is a major active component of *Tripterygium wilfordii* extracts, which are prepared as tablets and has been used clinically for the treatment of inflammation and autoimmune disorders. However, TP's therapeutic potential is limited by severe adverse effects. In a previous study, we reported that TP induced mitochondria dependent apoptosis in cardiomyocytes. Glycogen synthase kinase-3β (GSK-3β) is a multifunctional serine/threonine kinase that plays important roles in the necrosis and apoptosis of cardiomyocytes. Our study aimed to investigate the role of GSK-3β in TP-induced cardiotoxicity. Inhibition of GSK-3β activity by SB 216763, a potent and selective GSK-3 inhibitor, prominently ameliorated the detrimental effects in C57BL/6J mice with TP administration, which was associated with a correction of GSK-3β overactivity. Consistently, in TP-treated H9c2 cells, SB 216763 treatment counteracted GSK-3β overactivity, improved cell viability, and prevented apoptosis by modulating the expression of Bcl-2 family proteins. Mechanistically, GSK-3β interacted with and phosphorylated cyclophilin F (Cyp-F), a key regulator of mitochondrial permeability transition pore (mPTP). GSK-3β inhibition prevented the phosphorylation and activation of Cyp-F, and desensitized mPTP. Our findings suggest that pharmacological targeting of GSK-3β could represent a promising therapeutic strategy for protecting against cardiotoxicity induced by TP.

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1. Introduction

Tripterygium wilfordii Hook f. (TWHF), a traditional Chinese medicine (TCM), is attracting worldwide attention since the 1960s because its extracts were reported to be highly effective for rheumatoid arthritis (Tu, 2009; Zhou et al., 2014). More than three TWHF preparations have been approved by the China Food and Drug Administration (CFDA) for the therapy of autoimmune and inflammatory diseases, including rheumatoid arthritis, systemic lupus erythematosus, and ankylosing spondylitis (Ji et al., 2010). Triptolide (TP), the first identified diterpenoid triepoxide lactone, has been recognized as a principal ingredient responsible for the biological activities of TWHF (Kupchan et al., 1972). Moreover, TP has been shown to exert biological effects on tumor inhibition, the central nervous system, immuno-regulation and the cardiovascular system. However, the clinical applications of TP are limited by its narrow therapeutic window and severe toxicity (Li et al., 2014a, 2014b). The CFDA issued a warning in April 2012 about this medicine, urging caution. It was reported in clinical cases that this therapeutic drug elicited acute cardiogenic shock caused by myocardial damage, showing hydropic degeneration of myocardial cells, even with obvious contraction band necrosis in the papillary muscles (Chou et al., 1995; Huang et al., 2009).

Several research groups have reported the cardiotoxicity of TP, but the mechanism of TP-induced cardiac injury has not been fully elucidated. Our previous studies demonstrated that TP caused injury in H9c2 cells and BALB/C mice through oxidative stress and mitochondria-mediated apoptosis (Zhou et al., 2014). Reactive oxygen species (ROS) can trigger the opening of mitochondrial permeability transition pore (mPTP), resulting in the release of pro-apoptotic proteins such as cytochrome *c* and apoptotic protease-activating factor 1 (APAF1), which play a critical role in cell death (Jaeschke et al., 2012). The opening threshold of the mPTP and the sensitivity of mPTP to ROS are determined by the activation status of the mitochondrial membrane proteins like cyclophilin F (Cyp-F) (also known as mitochondrial cyclophilin D [Cyp-D]), which is regulated by a myriad of signaling pathways

Abbreviations: Cyp-F, cyclophilin F; DCF-DA, 2′, 7′-dichlorofluorescein-diacetate; GSK-3β, glycogen synthase kinase 3β; mPTP, mitochondrial permeability transition pore; PI, propidium iodide; ROS, reactive oxygen species; SB, SB 216763; SPF, Specific pathogen free; TP, triptolide; TWHF, *Tripterygium wilfordii* Hook f.; TCM, Traditional Chinese Medicine.

* Corresponding authors at: School of Pharmaceutical Sciences, 132 East Circle at Higher Education Mega Center, Guangzhou 510006, PR China.
E-mail addresses: shenfh3@mail.sysu.edu.cn (F. Shen), hzhiying@mail.sysu.edu.cn (Z. Huang).

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(Baines et al., 2005). Glycogen synthase kinase-3β (GSK-3β) was assumed to be a therapeutic target for cardiomyocyte protection, because it plays a crucial role in transferring regulatory signals downstream to modify the susceptibility to mPTP (Miura and Miki, 2009).

GSK-3β is one main isoform of a multifunctional serine/threonine kinase GSK-3, which was initially described as a key enzyme involved in glycogen metabolism, but is now known to regulate a diverse array of cell functions on cellular structure, growth, motility, metabolism and survival (Cohen and Frame, 2001). GSK-3β plays critical roles in the regulation of multiple signaling pathways, including canonical Wnt/wingless, insulin signaling, NF-κB and apoptotic signaling. As a key regulator of cellular fate, GSK-3β participates in a number of apoptotic signaling pathways by phosphorylating transcription factors that regulate apoptosis (Jope and Johnson, 2004). GSK-3β promotes apoptosis by both activating pro-apoptotic factors such as p53 and inactivating survival-promoting factors through phosphorylation (Grimes and Jope, 2001; Watcharasit et al., 2002). Further, inhibition of GSK-3β confers protection against myocardial ischemia reperfusion injury and doxorubicin-induced cardiomyocyte death (Miura and Tanno, 2010; Venkatesan et al., 2010).

Accumulative evidences indicate that GSK-3β is pivotal in determining cardiomyocyte death. Nevertheless, its role in TP-elicited cardiac injury remains unknown. Herein, we explored the effects of SB 216763, a potent and selective inhibitor of GSK-3, on the toxicity induced by TP in rat cardiac H9c2 cell lines and C57BL6/J mice. Indeed, our results demonstrated a critical protective role of GSK-3β inhibition in regulating myocardial cell apoptosis, revealing the clinical potential of GSK-3β inhibition for TP-induced acute heart injury.

2. Materials and methods

2.1. Materials and reagents

TP (>99% purity) was purchased from Shanghai DND Pharm-Technology Co. (Shanghai, China). SB 216763 (>98% 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 MP Biomedicals (Santa Ana, CA, USA). FITC Annexin V apoptosis detection kit and PE rabbit anti-active Caspase-3 were purchased from BD Biosciences (San Jose, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from HyClone (GE Healthcare, Chicago, IL, USA). Anti-phospho-Glycogen synthase antibody, anti-phospho-GSK-3β (Ser9) antibody, anti-GSK-3β antibody, anti-p53 antibody, anti-Bcl-2 antibody, anti-Bax antibody, anti-Caspase-3 antibody, anti-GAPDH antibody, and anti-β-actin antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Cyp-F and anti-phospho-serine antibody were purchased from Abcam Plc (Cambridge, UK). Other chemicals were of analytical grade from commercial suppliers.

2.2. Animals and treatment

Specific pathogen free (SPF) male C57BL6/J mice (6 weeks) were purchased from the Laboratory Animal Center of Sun Yat-sen University in Guangzhou, China. All mice were maintained under an SPF condition controlled at a temperature of 23 ± 3 °C and humidity of 55 ± 15%, with a 12-hour light-dark cycle. Food and water were provided ad libitum. All the animal experimental procedures in this study were performed in accordance with the protocol approved by the Animal Ethical and Welfare Committee of Sun. Yat-sen University (Approval No: IACUC-DD-16-0302).

To examine the role of GSK-3β in TP-induced cardiotoxicity, 24 mice were randomly assigned into the following treatment groups ($n = 6$ mice/group): (1) control group: mice were given intravenous (*i.v.*) injection of normal saline (the solvent for TP and SB 216763); (2) TP group: mice were treated with 1.2 mg/kg TP *i.v.* for one injection; (3) SB 216763 + TP group: mice were injected with 30 mg/kg SB 216763 intraperitoneally (*i.p.*) followed by 1.2 mg/kg TP 2 h later; and (4) SB 216763 group: mice were treated with 30 mg/kg SB 216763 *i.p.* injection. All mice were anesthetized using 20% urethane *i.p.* injection 24 h after TP injection, and blood samples were collected for serum biochemical assays. The hearts were removed and weighed. Half of the heart was fixed in 10% formalin for the histopathological examination, and the remaining part was used for the biochemical analysis.

2.3. Serum biochemical assay

The physiological functions were evaluated with serum levels of lactate dehydrogenase (LDH), aspartate transaminase (AST) and creatine kinase (CK). These parameters were measured by a CX5 automatic analyzer (Beckman Coulter, Brea, CA, USA) using standardized commercially available kits (Beijing Leadman Biochemistry Co., Beijing, China).

2.4. Histopathological examinations

The hearts were individually excised and immediately immersed in a formaldehyde solution for 24 h. The recipe for formaldehyde solution is 10% of 37–40% formaldehyde solution and 90% of 0.01 mol/L pH 7.4 PBS. After fixation and paraffin-embedding, 3-μm-thick sections were cut and stained with hematoxylin and eosin for overall morphological evaluation using an optical microscope (Leica DM5000B, Leica Camera, Wetzlar, Germany).

2.5. Cell culture and drug dissolution

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 in a humidified incubator under 5% CO_2 at 37 °C. Cell culture materials were purchased from NEST Biotech (Nest Biotechnology, Jiangsu, China).

TP was dissolved in DMSO to make stock solutions and stored at −20 °C. Stock solutions were freshly diluted in cell culture media or normal saline to the desired concentrations before use. SB 216763, dissolved in DMSO to make stock solutions, was freshly diluted in cell culture media or suspended in normal saline to the desired concentrations before use. DMSO content within the media never exceeded 0.1% (v/v).

2.6. MTT assay

H9c2 cells were seeded in a 96-well plate at a density of 4×10^3 cells/well. Twenty-four hours later, the cells were treated with control (0.1% DMSO) or SB 216763 (2 or 5 μM). Two hours later after SB 216763 pretreatment, the cells were exposed with TP solution (160 nM) for 24 h. After treatment, the cells were incubated with 5 mg/mL MTT tetrazolium (20 μL/well) for 4 h at 37 °C. The 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; Thermo Labsystems, Helsinki, Finland) (Li et al., 2012).

2.7. Determination of apoptosis

H9c2 cells were seeded in 6-well plates at a density of 4×10^5 cells/well. Consistent with treatment in MTT assay as previously described, the cells were incubated with control (0.1% DMSO) or TP (160 nM) for 24 h after pretreatment with SB 216763 (2 or 5 μM) 2 h later. Stained cell suspension was prepared to determine cell apoptosis through BD Accuri™ C6 cytometer using an Annexin V FITC apoptosis detection kit (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

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