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Original article

Effects of Ganoderma lucidum polysaccharides against doxorubicin-induced cardiotoxicity

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ABSTRACT

Doxorubicin (DOX) is a widely used anthracycline derivative anticancer drug, but the use of DOX in clinical applications is limited by its cardiotoxicity. In the current research, we were aiming to assess the effects of *Ganoderma lucidum* polysaccharides (GLPS) on DOX-induced cardiotoxicity and to illustrate the associated mechanisms. H9c2 rat cardiomyocytes were treated with DOX in the absence or presence of GLPS, and we found GLPS treatment ameliorated DOX-induced H9c2 cell death. Moreover, results of *in vivo* studies indicated that GLPS significantly decreased the serum levels of lactate dehydrogenase (LDH), creatine kinase (CK) and aspartate aminotransferase (AST) and attenuated DOX-induced histological changes of the heart tissues. In addition, we found DOX administration promoted myocardial apoptosis, potentiated oxidative stress, decreased the activities of antioxidant enzymes and increased the production of pro-inflammatory cytokines. However, GLPS pretreatment markedly attenuated all these untoward effects of DOX. Furthermore, GLPS pretreatment was found to inhibit Cul3-mediated K48-linked polyubiquitination of Nrf2 through suppressing Cul3 expression, thereby stabilizing Nrf2 expression in H9c2 cells after DOX treatment, leading to the decreased expression of P53 and p-P65 and increased levels of MDM2 and HO-1, resulted in the attenuated apoptosis, oxidative stress and inflammation induced by DOX.

1. Introduction

Doxorubicin (DOX) (also referred to as Adriamycin), an anthracycline antibiotic, is well known as one of the most widely-used chemotherapeutic drugs which is used for the treatment of various cancers [1–3]. However, the clinical application of DOX has also been limited because of its potential to induce the dose-dependent cardiotoxicity [4,5]. Elucidating the mechanisms underlying cardiotoxicity induced by DOX may identify strategies to reduce cardiomyopathy risks for cancer patients. Though the specific mechanisms of DOX-induced cardiotoxicity still remain largely unknown, many studies reported that oxidative stress, cardiomyocyte apoptosis and inflammation could play important roles in DOX-induced cardiotoxicity [6–9].

Ganoderma lucidum (Reishi or Ling-Zhi) is a basidiomycete white rot fungus which is widely used in the treatment of a variety of human diseases in China for thousand years [10–12]. A variety of bioactive substances have been extracted from *Ganoderma lucidum*, and *Ganoderma lucidum* polysaccharides (GLPS) have been shown to be the most important material responsible for its bioactivity [13,14]. Recently, GLPS was reported to play anti-tumor, anti-oxidation, anti-virus, anti-inflammation, anti-diabetes and neuroprotection roles in various physiological processes [15–19]. However, the effect of GLPS on DOX-induced cardiotoxicity still remains largely unknown.

The transcription factor NF-E2-related factor 2 (Nrf2) is one of the most important transcription factor to regulate the transcription of antioxidative genes such as heme oxygenase-1 (HO-1) [20]. Murine-double minute 2 (MDM2) was also reported as a target gene of Nrf2, the increased expression of MDM2 induced by Nrf2 was found to contribute to the down-regulation of P53, leading to stable mitochondrial membrane potential, reduced cytochrome c release as well as inhibited caspase-3 activation therefore suppressed mitochondrial apoptosis [21]. In response to inflammatory stimuli, up-regulation of Nrf2 signaling was also found to inhibit the overproduction of cytokines and chemokines, and limited the activation of NF-κB signaling pathway

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[22]. Recently, Nrf2 was reported as an endogenous suppressor of DOX-induced cardiotoxicity by controlling both oxidative stress and autophagy in heart tissues, indicated that chemical compounds or natural pharmaceutical ingredients that are able to activate Nrf2 will be potential candidate drugs with which to treat DOX-induced cardiotoxicity [23,24].

In this research, the effect of GLPS on DOX-induced cardiotoxicity was investigated. We found GLPS treatment ameliorated cell death, mitochondrial apoptosis, oxidative stress and pro-inflammatory cytokines production in DOX-treated rats and H9c2 cells. Interestingly, we also observed that GLPS could reverse the decreased expression of Nrf2 induced by DOX, promoting Nrf2 stabilization by suppressing Cul3-mediated K48-linked polyubiquitination of Nrf2, leading to the increased expression of MDM2 and HO-1, suppressing the activation of NF-κB signaling pathway. In conclusion, we suggested that GLPS as a potential candidate drug for the treatment of DOX-induced cardiotoxicity.

2. Materials and methods

2.1. Reagents

GLPS was obtained from Johnsun Mushroom (Hangzhou, China) and prepared as described [19]. DOX was purchased from Sigma-Aldrich (Sigma-Aldrich, MO, USA). The Cell Counting Kit-8 (CCK8) was obtained from Dojindo Laboratory (Japan). LDH, CK, AST, TNF-α, IL-6, IL-10 and MDA were measured by the kits purchased from BioLegend (San Diego, CA, USA). The commercial kits for detecting activities of the superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The caspase-3 activity assay kit was obtained from BioVision (Palo Alto, CA, USA) and Annexin V/PI apoptosis detection kit was produced by KEYGEN (Nanjing, China).

2.2. Rats and cells

Male Sprague-Dawley (SD) rats weighing 220 g–250 g were purchased from the Animal Center of Peking University Health Science Center (Beijing, China). The experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals approved by the Animal Ethics Committee of the Scientific Investigation Board of Chengde Medical University. Rat cardiomyocytes H9c2, human breast cancer cells MCF-7, human hepatocellular carcinoma cells HepG2 were obtained from ATCC (Manassas, VA). All the cells were cultured at 37° C under 5% CO_2 in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and 2 mM L-glutamine.

2.3. Experimental protocols

The rats were randomly divided into five groups: control group, GLPS (high-dose) group, DOX group, GLPS (low-dose) +DOX group, GLPS (high-dose) +DOX group (n = 15 per group). The rats in DOX-treated groups were dosed intraperitoneally with DOX (dissolved in saline, 2 mg/kg every other day for a total of 3 times). All rats received daily oral gavage with distilled water or GLPS (dissolved in distilled water, high dose: 100 mg/kg, low dose: 50 mg/kg) for 15 days prior to DOX. After 10 days of the final administration of DOX, the rats were sacrificed followed by the measurement of the body and heart weights. The heart tissues and blood samples were collected for histopathological examination, biochemical measurements and mechanism researches. For all *in vitro* experiments, the cells were plated at an appropriate density according to the experimental design, 24 h later, the cells were pretreated with distilled water or GLPS (dissolved in distilled water, high dose: 100 μg/ml, low dose: 50 μg/ml). Eight hours later, cells were treated with our without DOX (1 μM) for 16 h, and the cells were harvest for further studies.

2.4. In vitro cell viability and anti-tumor activity

Cell viability and anti-tumor activity was detected by CCK8 assay according to the manufacturer's instructions as previously reported [25]. Briefly, cells were cultured in a 96-well plate, each well contained 1×10^4 cells in a total volume of 100 μL. After GLPS or DOX treatment, 10 μL CCK-8 was added in each well, and the optical density (OD) value was detected at 450 nm after incubating at 37 °C for 2 h.

2.5. Biochemical Assays

In vivo, at the end of the experiment period, the serum samples of the rats were obtained and followed by the biochemical assays to examine the levels of LDH, CK and AST. The heart tissues were also collected to detect the content of MDA and activities of SOD, GSH, and CAT according to the kit manufacturer's instructions. *In vitro*, the cell culture medium of H9c2 cells was collected for the measurement of LDH release. Moreover, the cells were harvested followed by ultrasonication. After centrifugation for 5 mins at 1000*g*, supernatants were collected and the levels of MDA and activities of SOD, GSH, and CAT were assessed by commercial kits.

2.6. Histopathologic analyses

At the end of the experiment period, the heart tissues were fixed in 10% buffered formalin and embedded in paraffin. Approximately 5 μm thick sections were prepared from tissue paraffin block and stained with hematoxylin and eosin (H & E). After that, pathological changes were checked by light microscopy for observation of structural abnormality (200×). The severity of changes was quantitated based on the degree of inflammation, interstitial fibrosis and myocardial disorganization. The scoring system was as follows: 0 = no damage; 1 = minimal inflammatory cell infiltration, focal minimal interstitial fibrosis and mild myocardial disorganization (less than 5%); 2 = moderate inflammatory cell infiltration, patchy multifocal interstitial fibrosis and moderate myocardial disorganization (5–20%); 3 = widespread inflammatory cell infiltration, severe interstitial fibrosis and myocardial disorganization (more than 20%) [26].

2.7. Apoptosis assays

Apoptotic cells were counted by staining with Annexin-V and propidium iodide (PI). Flow cytometry was performed to detect the percent of apoptotic cells. The caspase-3 activity of cell extracts ($\sim 10^6$ cells) was examined by DEVD-AMC (Peptron) according to the manufacturer's instructions.

2.8. Quantitative PCR analysis and ELISA

Heart tissues or H9c2 cells were collected at the end of the experiment period. Quantitative PCR was performed as previously reported [27]. GAPDH was used as the internal control. Primer sequences used in quantitative PCR were shown in Table 1. ELISA assays were performed to examine the production of TNF-α, IL-6, and IL-10 in serum or culture medium according to the manufacturer's instruction.

2.9. Western blot analysis

The protein extraction and western blot analysis was performed as previously described [28]. Ubiquitination assay was performed as described [27]. The antibodies for cleaved caspase-8, cleaved caspase-3, cleaved PARP, cytochrome c, MDM2, β-actin and ubiquitin (linkage-specific K48) were obtained from Abcam (Abcam, Cambridge, UK), and the other antibodies were purchased from Cell Signaling Technology