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Activation of AMPK-dependent SIRT-1 by astragalus polysaccharide protects against ochratoxin A-induced immune stress *in vitro* and *in vivo*

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ABSTRACT

Recent studies have highlighted the immune stress caused by ochratoxin A (OTA), but little attention was paid to its alleviation. In the present study, the protective effects of astragalus polysaccharide (APS) against OTA-induced immune stress *in vitro* and *in vivo* and its mechanism/(s) involved were investigated. The *in vitro* results showed that APS (20 μg/ml) induced a significant decrease in cytotoxicity, apoptosis and pro-inflammatory cytokine expressions elevated by OTA (1.5 μg/ml) in porcine alveolar macrophages (PAMs). *In vivo*, APS (200 mg/kg b.w.) significantly alleviated OTA-induced (75 μg/kg b.w.) spleen damages and decreased the expressions of OTA-promoted apoptosis-related protein and pro-inflammatory cytokine. Further study indicated that APS caused significant enhancement of AMPK/SIRT-1 and inhibition of NFκB in PAMs and mice. The down-regulation of SIRT-1 by EX527 *in vivo* or EX527 and SIRT-1 knockdown *in vitro* abolished the inhibitory effects of APS on OTA-induced cytotoxicity, apoptosis, spleen damages and pro-inflammatory cytokine expressions. Taken together, these findings indicate that APS could attenuate the immune stress induced by OTA *in vitro* and *in vivo via* activation of the AMPK/SIRT-1 signaling pathway.

1. Introduction

Ochratoxin A (OTA) produced by the fungus *Penicillium* and *Aspergillus* is a frequent contaminant of food and feedstuffs in the world, which exerts plenty of toxic effects to several animal species and humans [1,2]. Among the farm animals, pig is one of the most sensitive species to OTA. Studies on pigs have indicated that the ingestion of OTA could induce metabolic disorders, leading to serious economic consequences [3,4]. Recently, OTA as an immunotoxin has become the focal point of research because of its slow elimination from body [5]. Macrophages play an important role in the first line of defense against invading pathogens, where they act as immune effectors and antigen presenting cells [6]. Porcine alveolus macrophages (PAMs) are the immune cells with many functions, which are the important objects in the study of cell phagocytosis, cellular immunity, and molecular immunology [7,8]. Our previous study has demonstrated that OTA can induce immune stress in PAMs by impacting cell proliferation, cytokine expressions and immune response through specific cell signaling activation [9]. However, little attention was paid to the alleviation of the immune stress induced by OTA.

Traditional herbal medicine has been widely applied in clinic to treat a variety of diseases as a supplemental or alternative medicine in many countries, especially Asian countries. Astragalus polysaccharide (APS) is the major active constituent of *Astragalus membranaceus* which is a commonly used Chinese medicinal plant. Scientific evidences showed that APS could exert extensive range of biological activities, including anti-oxidation and anti-inflammation [10–12]. Although APS has been proved to regulate immune responses *in vitro* and *in vivo* [13,14], no study related to the effects of APS on the immunoregulation in animals suffered from mycotoxin has been reported. Therefore, exploring the effects of APS on OTA-induced immune stress may be of great clinical importance in improving the immunity in animals suffered from mycotoxin by nutrition regulations.

Sirtuin 1 (SIRT-1), a highly conserved NAD^+-dependent class III histone/protein deacetylase, plays diverse role in senescence, reactions to oxidative stress, apoptosis, metabolism, and inflammation [15,16]. It exerts physiological functions by histones and transcription factors deacetylation, including nuclear factor-κB (NFκB) [17]. SIRT-1 activator resveratrol has been reported to protect the ovary against chromium-toxicity [18]. SIRT-1 has been proven to be a critical immune regulator by modulating macrophage functions [19]. Whether SIRT-1 activation plays a vital role for APS in regulating OTA-induced immune stress has not yet been elucidated.

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The purpose of the present study was to investigate the effects of APS on immune stress induced by OTA *in vitro* and *in vivo,* and the detailed mechanism involved.

2. Materials and methods

2.1. Chemicals

The APS (purity > 95%) used in cell experiments was purchased from Pharma Genesis, Inc. (USA). The APS (purity > 80%) used in animal experiments was purchased from Ci Yuan Biotechnology Co. Ltd. (Shannxi, China). The OTA used in our study was purchased from Sigma (USA).

Antibodies against β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, USA); phospho-AMPK, acetyl-p65 and SIRT-1 were purchased from Abcam (Abcam, USA); caspase-3, caspase-9, phospho-65 and secondary antibodies were purchased from Cell Signaling Technology (CST, USA). EX527 was purchased from Med Chem Express (MCE, USA).

2.2. Cell culture

PAM cell line 3D4/21 (ATCC, CRL-2843) was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences and cultured in Dulbecco's minimal Eagle's medium (DMEM; Gibco, USA) supplemented with 8% fetal bovine serum (FBS; Gibco, USA), 1% penicillin, 1% streptomycin and 1% MEM nonessential amino acids at 37 °C in 5% CO_2 atmosphere [9].

2.3. Animals and experimental design

Fifty-four Kunming mice were provided by the Center of Laboratory Animals, Yangzhou University (Yangzhou, China). Animals were singly housed in cages in animal quarters under constant conditions of light/dark cycle (12 h/12 h), temperature (23 ± 1 °C) and humidity (60 ± 10%) for 1 week prior to experimentation, and had free access to food (standard chow diet, Jiangsu, China) and tap water. All procedures involving mice were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD, USA) and approved by the Committee for the Care and Use of Experimental Animals at the Agriculture University of Nanjing (Certification No.: SYXK (Su) 2011–0036).

For the first randomized trial, mice were randomly divided into five groups (each group includes 6 mice): a normal control group, a OTA group and three OTA + APS groups with different doses (100, 200, 400 mg/kg b.w. APS). For the second randomized trial, mice were randomly divided into four groups (each group includes 6 mice): a normal control group, a OTA group, a OTA + APS group and a OTA + APS + EX527 group. Every day during the experimental period, the mice in the APS groups were administered APS in 0.5% CMC-Na by gavage. Other groups were given with equivalent amounts of PBS by gavage. On the sixth day the experiment, the mice from OTA group were injected intraperitoneally (i.p.) with 75 μg/kg b.w. every other day for 20 days [20]. The control group was injected intraperitoneally (i.p.) with equivalent amounts of PBS. The fourth group was given intraperitoneally 5 mg/kg b.w. of EX527 3 days before OTA treatment [21]. Other groups were given intraperitoneally with equivalent amounts of PBS.

2.4. Assessment of cell viability

PAMs were exposed to APS or OTA at different concentrations for 60 h and inflicted on the colorimetric 3 (4, 5 dimethylthiazol 2 yl) 2, 5 diphenyltetrazolium bromide (MTT) (Sigma, USA) assay. Absorbance was measured at 490 nm with a reference wavelength of 595 nm.

2.5. Measurement of lactate dehydrogenase (LDH) release

The supernatants were collected into tubes and stored at −80 °C until assay. LDH release was determined using lactate dehydrogenase assay kit (Jiancheng, China). One unit of enzyme activity was defined as equivalent to 1 mmol of reduced nicotinamide adenine dinucleotide oxidized per minute. The data were expressed as percentage of the control values.

2.6. Quantitative real-time PCR (qRT-PCR) analysis

The PCR primers designed and synthesized by Invitrogen were listed in Table 1. Total RNA was extracted from PAMs using trizol reagent (Life technologies, USA), dissolved in diethylpyrocarbonate-treated water, and then reversed transcribed using Oligo dT primers and MMLV reverse transcriptase (TaKaRa, China). The relative mRNA levels of target genes were determined using the Δ cycle threshold (ΔCt) method with β-actin serving as a reference gene.

2.7. Detection of morphological changes and annexin V/PI staining

Morphology of apoptotic cell nuclei was assessed by staining with the DNA binding fluorochrome Hoechst 33258. Briefly, PAMs were seeded in 12-well plates with corresponding treatment. After 60 h incubation, cells were fixed with 4% paraformaldehyde for 20 min at 4 °C. After three washes in PBS, cell nuclei were counterstained with Hoechst 33258 for 5 min and then examined by the fluorescence microscopy. The apoptotic cells were identified by condensation and fragments of nuclei.

Apoptosis was evaluated by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA, United States) using Annexin V/PI apoptosis detection kit (BD Pharmingen, USA) according to the manufacturer's protocol. Briefly, the harvested cells were resuspended with Binding buffer and stained with Annexin V-FITC and propidium iodide (PI). More than 10,000 cells were collected with FACS to assess the apoptosis rate.

2.8. Transfection

Specific siRNAs (SIRT-1-specific siRNA, 5′ GCUGAUGAACCACUUGCUA TT 3′; control siRNA, 5′ UUCUCCGAACGUGUCACGUTT 3′) were designed and synthesized by Invitrogen BlockiT RNAi designer. Transfection was performed as described previously [22] with a minor modification. Briefly, PAMs were cultured in DMEM with 8% FBS and then transfected with siRNAs using X-treme GENE Transfection Reagent (as the manufacturer's protocol; Roche, Basel, Switzerland) for 4–6 h. The cells were then washed and transferred to DMEM with 4% FBS for various treatments.

2.9. Western blot

Total protein was obtained from the lysed cells and tissues. Equal amounts of lysates were submitted to SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, USA). After blocking with 5% non-fat milk for 2 h, the membranes were

Table 1
Primers used for quantitative real-time PCR.

Gene	Accession no.	Primer sequence (5′-3′)	Product (bp)
β-actin	DQ845171.1	Forward: CTGCGGCATCCACGAAACT Reverse: AGGGCCGTGATCTCCTTCTG	147
IL-1α	NM_214029.1	Forward: CCAATGACACAGAAGAAG Reverse: CCAGGTTATTTAGCACAG	183
IL-1β	NM_214055.1	Forward: CCCAAAAGTTACCCGAAGAGG Reverse: TCTGCTTGAGAGGTGCTGATG	125
IL-6	NM_214399.1	Forward: CCAGGAACCCAGCTATGAAC Reverse: CTGCACAGCCTCGACATT	446
TNF-α	JF831365.1	Forward: GACTCAGATCATCGTCTC Reverse: GGAGTAGATGAGGTACAG	179