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X-11-5-27, a daidzein derivative, inhibits NLRP3 inflammasome activity via promoting autophagy

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ABSTRACT

NLRP3 inflammasome is a cytoplasmic multiprotein complex which plays a critical role in response to infection or injury, however, aberrant NLRP3 inflammasome activation is deleterious. In our study, we investigate the inhibitory effect of X-11-5-27, a daidzein derivative, on the NLRP3 inflammasome. The results showed that the activation of NLRP3 inflammasome was inhibited by X-11-5-27 in a dose-dependent manner, followed by a decrease in the cleavage of caspase-1 and maturation of IL-1β. Furthermore, we found that X-11-5-27 significantly restrained the formation of NLRP3 inflammasome. At the same time, X-11-5-27 time- and dose-dependently decreased the production of ROS and superoxide. In addition, X-11-5-27 enhanced the activity of SOD to scavenge ROS release. This inhibitory effect of X-11-5-27 was due to the protection of mitochondrial homeostasis and was abolished after the treatment of rotenone. Notably, X-11-5-27 was found to trigger autophagy in macrophages, which in turn inhibited the NLRP3 inflammasome activation. Moreover, the phosphorylation states of the proteins in PI3K/AKT/mTOR signaling pathway were dramatically decreased after X-11-5-27 treatment. In conclusion, our results demonstrate that autophagy-mediated ROS reduction is responsible for X-11-5-27-induced NLRP3 flammasome inactivation. And these results may help guide decisions regarding the use of X-11-5-27 in relieving the inflammasome-driven hyper-inflammation.

1. Introduction

Inflammation is an important part of the immune system to maintain cellular homeostasis via defending infection or injury. However, aberrant inflammation often results in tissue damage and organ failure mediated by uncontrolled inflammasome activation and the up-regulation of pro-inflammatory enzymes [1]. Importantly, it has been noted that excessive inflammasome activation is associated with a number of human disease, such as metabolic syndrome and inflammatory bowel disease (IBD) [1,2]. Furthermore, mutations of inflammasome complexes have been proved to induce activation of caspase-1 and secretion of IL-1β, a pro-inflammatory cytokine involved in both acute and chronic inflammatory responses [2]. NLRP3 is the best-characterized NLR family member, which senses microbial ligands in the cytosol to trigger the assembly of a large multi-protein complex named the Nlrp3 inflammasome. Once activated, NLRP3, the ASC adaptor, and pro caspase-1 will form the multi-protein complex and then activates caspase-1 to cleavage IL-1β and IL-18 [3,4]. So far, the activation of the NLRP3 inflammasome is now considered as a two-step process which requires priming and activation [5]. The first step, "priming" transcriptional step, involved the NF-κB-dependent transcription of NLRP3 and pro-IL-1β (signal 1). Then, many of the stimulators, including K^+ efflux, mitochondrial dysfunction or lysosomal rupture, promote the assembly and activation of the NLRP3 inflammasome (signal 2) [6].

It is imperative to know that ROS, produced by mostly all NLRP3 agonists, is shown to be closely related to NLRP3 inflammasome activation in response to many inflammatory stimuli [6]. However, the role of ROS is still controversial in the regulation of NLRP3 inflammsome. Numerous studies show that ROS are essential for priming or activation of NLRP3 inflammasome [7,8]. Initially, NLRP3 priming requires a ROS-sensitive proinflammatory signal pathway which could be blocked by ROS scavenger, such as NF-κB and MAPK [9–11]. Studies by Bauernfeind, F and Liu X also demonstrate that ROS blockade by

Abbreviations: NLR, NOD-like receptor; IL-1β, interleukin-1β; ASC, apoptosis-associated speck-like protein which contains a CARD; NF-κB, nuclear factor-κB; BMDM-Ms, Bone marrow derived macrophages cells; THP-Ms, THP-1 cell-derived macrophages; PMA, Phorbol myristate acetate; LPS, lipopolysaccharide; ATP, adenosine triphosphate; ROS, reactive oxygen species; SOD, superoxide dismutase; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; mtROS, mitochondrial reactive oxygen species; mtDNA, mitochondrial DNA; mTOR, mammalian target of rapamycin

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chemical antioxidants suppresses the priming of NLRP3 inflammasome, but not the activation [8,12]. Recently, it is found that ROS regulates the post-translational activation of NLRP3 by stimulating its deubiquitination. This progress is dependent on mitochondrial reactive oxygen species (mtROS) and can be reversed by ROS scavengers [13]. In another study, mitochondrial damage is reported to activate the NLRP3 inflammasome through mtROS [6]. Furthermore, mitochondrial DNA (mtDNA) has also been reported to directly bound to and cause activation of NLRP3 inflammasome in programmed cell death [5]. Consistent with these studies, mitochondrial events appear to play pivotal roles in NLRP3 inflammasome activation.

Autophagy is an evolutionarily conserved, multi-step lysosomal degradation progress for maintaining cell homeostasis [14,15]. A number of evidences indicate that autophagy plays a critical role in response to extracellular and intracellular stress, especially during starving [16,17]. Furthermore, autophagy has also been confirmed to participate in the removal of ROS produced after oxidative stress in different types of somatic cells [17]. Other evidence has been raised to suggest the stability of autophagy is essential for maintaining the function of cells, as well as for removing the toxic molecules produced during oxidative stress [17,18]. Recently, mitochondria-selective autophagy, termed mitophagy, has emerged as a central player in maintaining mitochondrial homeostasis and modulating NLRP3 inflammasome activation. Moreover, mitophagy has also been reported to inhibit NLRP3 inflammasome through the elimination of mtDNA and mtROS [19]. Besides, increasing evidence indicates that mammalian target of rapamycin (mTOR) is a key regulator of autophagy [20].

Daidzein is an isoflavone isolated from a number of plants and food sources [21]. It's a conventional herbal medicine which exerts various pharmacological properties, such as anti-oxidant, anti-inflammation and anti-cancer [21,22]. Recent evidences indicate that daidzein can suppress NF-κB-induced expression of IL-6 in fibroblasts and macrophages [23]. It is reported to inhibit high glucose-induced vascular complications in diabetes through blocking indirect nitric oxide formation and scavenging ROS generation [24]. Therefore, twenty derivatives of daidzein were designed and synthesized, and among them, X-11-5-27 exhibits much more potent anti-inflamamtion activity compared with others. The goal of the present study is to evaluate the inhibitory effect of X-11-5-27 on NLRP3 inflammasome and the relevant mechanism.

2. Materials and methods

2.1. Reagents

X-11-5-27 was synthesized in Organic Chemistry Laboratory of China Pharmaceutical University (Nanjing, China). Samples containing X-11-5-27 at a minimum of 99% purity were used for the experiments unless otherwise indicated. Primary antibodies against β-actin, IL-1β, horseradish peroxidase (HRP) conjugated second antibodies were purchased from Bioworld Technology Inc. (CA, USA). NLRP3 and caspase1 antibodies were from Abcam Technology Inc (MA, USA). LC3 A/B, p-mTOR, p-AKT and p-PI3K were purchased from Cell Signaling Technology (Danvers, USA). PMA, MTT, LPS and ATP were obtained from Sigma-Aldrich (MO, USA). ELISA kit for human IL-1β was purchased from Boster Biotech Co. Ltd. (Wuhan, China). Immunohistochemistry kit was purchased from KeyGEN Biotech Inc (Nanjing, China). 3-methyladenine (3-MA) was purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488 goat anti-rabbit IgG was purchased from Keygen Biotech (Nanjing, China). MitoTracker Red CMXRos was purchased from Invitrogen (MA, USA).

2.2. Cell culture

Human acute monocytic leukemia THP-1 cells and L929 cells were obtained from Cell Bank of the Chinese Academic of Sciences (Shanghai, China). THP-1 and L929 cells were cultured in RPMI-1640 medium and DMEM, respectively, and supplemented with 10% fetal bovine serum (Gibco, NY, USA). Bone marrow derived macrophages cells (BMDMs) were isolated following the procedures below. Bone marrow cells were isolated from femur and tibia of 4–6 week-old C57BL/6 mice and cultured with cell-conditioned medium supplemented with 10% fetal bovine serum and 30% L929 supernatant. Conditioned medium was changed every 3 days. Under these conditions, adherent macrophages were obtained after 7 days. Non adherent cells were removed by washing the plate with PBS. The adherent macrophages were used for the experiment. All cell lines were incubated under a humidified 5% (v/v) CO_2 atmosphere at 37 °C.

2.3. Western blot assay

After washing twice with PBS, cells were collected and lysed in lysis buffer (100 mM Tris–Cl, pH 6.8, 20% (v/v) glycerol, 4% (m/v) SDS, 1 mM PMSF, 200 mM β-mercaptoethanol, 0.1 mM DTT and 1 mM NaF) for 1 h on ice. The lysates were separated by centrifugation (13,000 rpm) at 4 °C for 20 min. Protein concentrations were quantified with BCA protein (Thermo, MA, USA). All extracts were diluted in 1:3 ratio with sample buffer and boiled for 8 min at 100 °C. Protein extracts were quantitated to ensure subsequent same loading. Protein samples were separated by 12% SDS-PAGE and transferred onto the PVDF membranes (Millipore, MA, USA). Then the blots were hybridized with specific antibodies of NLRP3, caspase-1, IL-1β, β-actin and lamin A overnight at 4 °C. After washing, blots were exposed to HRP-conjugated secondary antibodies for 1 h at 37 °C. All of the antibodies were diluted by PBST with 1% BSA. Enhanced chemoluminescent reagents (Beyotime, Jiangsu, China) were used to detect the proteins on the immunoblots. The protein bands were quantified by using Quantity One software (Bio-rad, CA, USA), the results showed the relative amount of protein that normalized to β-actin.

2.4. ASC oligomerization assay

THP-Ms were seeded in 6-well plates. After the treatment with indicated stimuli, cells were washed by cold PBS and resuspended in an ice-cold buffer (Buffer A: 20 mM HEPES-KOH, pH 7.5, 150 mM KCl, 1% NP-40, 0.1 mM PMSF, and protease inhibitor), and lysed by shearing 10 times through a 21-gauge needle. Nuclei and unlysed cells were removed by centrifugation at 250 g for 5 min. The cell lysates were then centrifuged at 5000 g for 10 min at 4 °C. After washing twice with PBS, the pellets were crosslinked with fresh DSS (2 mM) for 30 min at 37 °C. The crosslinked pellets were separated in 12% SDS-PAGE and immunoblotting was performed.

2.5. Measurement of intracellular ROS level and cellular superoxide

Reactive Oxygen Species Assay kit purchased from Beyotime Institute of Biotechnology (Nanjing, Jiangsu, China) was used according to the manufacturer's instructions. Cells were cultured in a 12-well plate, and treated with various stimulations, then the cells were harvested and incubated with 100 μM DCFH-DA attenuated with serum-free medium for 20 min at 37 °C in the dark, washed twice with cold PBS. The fluorescence intensity was measured by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA) at Ex. / Em. = 488 / 525 nm.

2.6. Measurement of production of cellular superoxide

THP-Ms superoxide levels were measured with dihydroethidium (DHE, Beyotime Institute of Biotechnology, Nanjing, China), X-11-5-27 pretreated THP-Ms suffered different stimulations and were stained by DHE (10uM, 30 min, 37∘C) in dark in a humidified chamber, briefly washed by PBS and detected by FACS.

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