Epimedin B Through Enhancing NLRP3 Inflammasome Activation To Induces Idiosyncratic Hepatotoxicity

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Research

Keywords: Epimedii folium, Epimedin B, Idiosyncratic drug-induced liver injury, NLRP3 inflammasome

DOI: https://doi.org/10.21203/rs.3.rs-810002/v1

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Abstract

**Background:** The NLRP3 inflammasome plays a crucial role in the pathogenesis of various human diseases, also idiosyncratic drug-induced liver injury (IDILI). Epimedii Folium (EF) is commonly used for treating bone fractures, joint diseases and some chronic illness, but the EF also could induce IDILI. Several studies have confirmed that EF may induce liver injury by upregulating the activity of the NLRP3 inflammasome. However, the major active constituents of EF have not been well-studied.

**Results:** In the present study, we showed that epimedin B, a major active ingredient of EF, induced the development of IDILI by promoting the activation of the NLRP3 inflammasome. Synergistic induction of mitochondrial reactive oxygen species was a crucial contributor to the promoting effect of epimedin B observed on nigericin- or ATP-induced NLRP3 inflammasome activation. Importantly, epimedin B induced liver injury in the LPS-mediated susceptibility mouse model of IDILI while specific NLRP3 inhibitor MCC950 pretreatment completely abrogated the Caspase-1 activation and IL-1β secretion then couldn't induce liver injury.

**Conclusions:** Epimedin B specifically facilitated nigericin- or ATP-induced NLRP3 inflammasome activation and the development of IDILI, which is responsible for EF-induced liver injury. These findings suggest that epimedin B is one of the key constituents of liver injury caused by EF; the content of epimedin B in EF may be a risk factor for IDILI, especially in patients with diseases related to nigericin- or ATP-induced NLRP3 inflammasome activation.

Background

Idiosyncratic drug-induced liver injury (IDILI) is an uncommon but challenging clinical problem with respect to both diagnosis and management[1–4]. IDILI is unpredictable, not dose-dependent, and cannot be easily reproduced in animal models[5]. Recently, with the widespread use of herbal and dietary supplements (HDS) worldwide, traditional Chinese medicine (TCM) and dietary supplements have gained prominence as the leading causes of IDILI, not only in Asia but also in the United States and Europe[6, 7]. Specifically, the incidence of IDILI induced by TCMs, such as Polygonum multiflorum, Epimedii Folium, Psoraleae ructus, and Dictamni ortex, has increased in recent years[8–11]. However, its precise pathogenesis remains elusive.

The NLRP3 (the nucleotide-binding domain and leucine-rich repeat (NLR) pyrin domain containing inflammasome is a multiple protein complex consisting of NLRP3, apoptosis-associated specklike protein containing CARD (ASC), and cysteinyl aspartate-specific proteinase-1 (caspase-1), which can be activated by pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs)[12, 13]. Upon activation, it leads to the cleavage of pro-caspase-1, subsequently resulting in pyroptosis and the production of interleukin 1β (IL-1β) and IL-18. Aberrant activation of the NLRP3 inflammasome plays a crucial role in the pathogenesis of many chronic and degenerative diseases, such as Alzheimer's disease, osteoarthritis, type 2 diabetes, gout, atherosclerosis, and liver disease[14–16].
Activation of the NLRP3 inflammasome may be a critical mechanism underlying the development of IDILI\textsuperscript{[14, 15, 17]}. 

Epimedii Folium (EF) is a widely used herbal medicine in many countries and has been extensively used as a tonic or antirheumatic agent in clinics. Nevertheless, EF and its preparations have garnered significant interest because they can induce liver injury\textsuperscript{[10, 17–19]}. The major active constituents of EF are flavonoids. More than 60 kinds of flavonoids have been identified, of which epimedin A, B, C, and icariin are considered major bioactive components that constitute more than 52\% of the total flavonoids in EF\textsuperscript{[20–22]}. However, the underlying molecular mechanisms of the major constituents of EF about liver injury remains unclearly. In this study, we have demonstrated that epimedin B, which is one of the major constituents derived from EF, specifically promotes the activation of the NLRP3 inflammasome to induce IDILI.

**Materials And Methods**

**Mice**

Female wild-type C57BL/6 mice (age: 6–8 weeks) were obtained from SPF Biotechnology Co., Ltd. (Beijing, China). Nlrp3 knockout (Nlrp3\textsuperscript{-/-}) mice were obtained from the National Center of Biomedical Analysis (NCBA, Beijing, China) supplied by Dr. Tao Li. All mice were maintained under a 12-h light/dark cycle at 22–24 °C with ad libitum access to food and water. All animal protocols in this study were performed according to the guidelines outlined for care and use of laboratory animals and approved by the Animal Ethics Committee of the Fifth Medical Centre, Chinese People’s Liberation Army General Hospital (animal ethics committee approval No. IACUC-2017-003).

**Cell culture**

Bone marrow-derived macrophages (BMDMs) were isolated from the femoral bone marrow of 10-week-old female WT or Nlrp3\textsuperscript{-/-} female C57BL/6 mice and were cultured in the Dulbecco’s modified Eagle medium (DMEM) supplemented with 10\% fetal bovine serum (FBS), 1\% penicillin/streptomycin (P/S), and 50 ng/mL murine macrophage colony-stimulating factor (M-CSF). THP-1 cells were cultured in the RPMI 1640 medium supplemented with 10\% FBS and 1\% P/S. Cells were maintained in a humidified 5\% (v/v) CO\textsubscript{2} incubator at 37 °C.

**Antibodies and reagents**

Adenosine triphosphate (ATP), nigericin, SiO\textsubscript{2}, poly (deoxyadenylic-thymidylic) acid sodium salt (poly (dA:dT)), polynucleosinic: polynucleotidic acid [poly (I:C)], Pam3CSK4, dimethyl sulfoxide (DMSO), and LPS (Escherichia coli, 055: B5) were purchased from Sigma-Aldrich (Munich, Germany). Epimedin A (110623–72-8, purity 99.0\%), epimedin A1 (140147–77-9, purity 99.92\%), epimedin B (110623–73-9, purity 99.39\%), epimedin C (110642–44-9, purity 99.1\%), icariin (489–32-7, purity 97.64\%), caritin (118525–40-9, purity 99\%), icariside I (56725–99-6, purity 99.47\%), and anhydroicaritin (38226–86-7, purity 99.51\%).
were purchased from TargetMol. *Salmonella* strains were kindly provided by Dr. Tao Li from the National Center of Biomedical Analysis. MCC950 was obtained from TargetMol (Boston, MA, USA). Anti-mouse caspase-1 (1:1000, AG-20B-0042) was purchased from Adipogen (San Diego, CA, USA). Anti-mouse IL-1β (1:1000, 12,507) and anti-NLRP3 (1:2000, 15101S) antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). Anti-ASC (1:1000, sc-22,514-R) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-GAPDH (1:2000, 60,004–1-1 g) was purchased from Proteintech (Chicago, IL, USA). The color prestained protein marker (20AB01) was purchased from GenStar (Beijing, China).

**Inflammasome activation**

To induce inflammasome activation, BMDMs were seeded at a density of 5×10^5 cells/well in 0.5 mL of the medium in 24-well plates and were incubated overnight. Next, the medium was replaced with the fresh medium the following day, and BMDMs were subjected to stimulation with 50 ng/mL LPS or 1 μg/mL Pam3CSK4 for a duration of 4 h. The cells were subjected to treatment with Epimedin B for 1 h and were then stimulated as follows: 5 mM ATP for 1 h, 7.5 μmol/L nigericin for 30 min, or 250 μg/mL silicon dioxide (SiO2) for 6 h. Cells were transfected with poly (I:C) (2 μg/mL), poly(dA:dT) (2 μg/mL), or LPS (1 μg/mL) for 6 h using Lipofectamine 2000 according to the manufacturer's instructions.

**Western blotting**

Cell extracts and precipitated supernatants were subjected to lysis using 1×loading buffer containing radioimmunoprecipitation assay buffer. The samples were subjected to denaturation at 105 °C for 15 min. Equal amounts of protein samples were separated by performing 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and protein bands were transferred onto 0.2-mm polyvinylidene fluoride membranes. The membrane was blocked with 5% non-fat milk for 1 h at room temperature. Next, the indicated primary antibodies were added and the bands were incubated at 4 °C overnight and then subjected to treatment with anti-goat IgG (1:3000), anti-mouse IgG (1:5000), or anti-rabbit IgG (1:5000) for 1 h at room temperature. The signals generated thereafter were analyzed using an enhanced chemiluminescence reagent.

**Caspase-1 activity assay**

The Caspase-Glo® 1 Inflammasome Assay (Promeega, Madison, WI, USA) was used to assess caspase-1 activity in cell culture supernatants according to the manufacturer's instructions.

**Enzyme-linked immunosorbent assay (ELISA)**

Cell culture supernatants and mouse serum were analyzed for mouse IL-1β (R&D Systems, Minneapolis, MN, USA), IL-1β, TNF-α, and IL-6 (Dakewe, Beijing, China), respectively, according to the manufacturer's instructions.

**Alanine aminotransferase (ALT) and aspartate transaminase (AST)**
Serum ALT and AST levels were determined according to the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and the Nanjing Jiancheng Bioengineering Institute (GOT) assay kit instructions [23].

**Lactate dehydrogenase (LDH) assay**

The release of LDH into the culture supernatant was assessed using the CytoTox 96® 1 Non-radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

**ASC oligomerization**

Cells were subjected to lysis using Triton buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, and EDTA-free protease inhibitor cocktail). The samples were then centrifuged at 6,000 × g at 4°C for 15 min. The supernatant was referred to as Triton X-soluble, and the pellet fractions were referred to as Triton X-insoluble fractions. To enable ASC oligomer cross-linking, the Triton X-100-insoluble fractions were subjected to washing steps and were resuspended in 200 μL of PBS, followed by the establishment of cross-linking at 37°C with 2 mM disuccinimidyl suberate (DSS) for 30 min. The pellets were centrifuged at 6,000 × g for 15 min, after which they were collected and dissolved in 1×SDS loading buffer for immunoblot analysis.

**Intracellular K⁺ measurement**

BMDMs were seeded in 12-well plates overnight and were primed with 50 ng/mL LPS for 4 h. The cells were subjected to treatment with epimedin B and were then stimulated with nigericin for 30 min. The culture medium was thoroughly aspirated and subjected to washing steps thrice using potassium-free buffer. Ultrapure HNO3 was added to perform lysis of the cells. Samples were collected in glass bottles and boiled for 30 min at 100 °C. Intracellular K+ measurements were performed via inductively coupled plasma mass spectrometry.

**Measurement of intracellular Ca²⁺ levels**

BMDMs were seeded in a 384-well plate at a density of 2.5×10⁴ cells/mL overnight. Then, the cells were primed with LPS for 4 h, followed by stimulation with ATP for 45 min with or without epimedin B. A trace showing ATP-induced Ca²⁺ flux was analyzed using the FLIPRT Tetra system (Molecular Devices, San Jose, CA, USA).

**Mitochondrial reactive oxygen species assay**

BMDMs were seeded at a density of 1×10⁶ cells/mL in culture dishes with a diameter of 100 mm and primed with LPS (50 ng/mL) for 4 h. The cells were added in a test tube, subjected to washing steps with Opti-MEM, and were stimulated as per methods described previously. For mitochondrial reactive oxygen species (ROS) measurements, BMDMs were subjected to staining procedures using 4 μM MitoSOX for 20 min at 37 °C and then washing steps were conducted twice with HBSS, followed by assessments using
flow cytometry. After the completion of staining and washing procedures, flow cytometry was performed to measure mtROS levels.

**Hepatic mRNA expression**

RNA extraction from the liver tissue was performed using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, total RNA samples were reverse-transcribed into first-strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific). A total of 5 μL of the PowerUp SYBR Green Master Mix (A25742, Invitrogen), 3 μL DEPC water, 0.5 μL F primer, 0.5 μL R primer, and 1 μL cDNA were introduced into each well of a 384-well plate, and each sample was analyzed in triplicate via reverse-transcriptase quantitative PCR (RTqPCR).

**Assessment of the effects of LPS/epimedin B cotreatment-induced DILI in vivo**

C57BL/6 mice (6-8-week-old female) were subjected to starvation for 24 h and were administered with LPS (2 mg/kg) or saline vehicle via tail vein (i.v.). Following an observation period of 2 h, Epimedin B (20 mg/kg, 40 mg/kg, and 80 mg/kg) or its vehicle was administered via intraperitoneal injection. Mouse serum and a fraction of liver tissues were collected 6 h after epimedin B treatment. Serum ALT, AST, IL-1β, and TNF-α levels were measured. Histopathological analysis was performed via hematoxylin and eosin (H&E) staining. F4/80-positive macrophages in the liver were also estimated.

In the second experiment, female C57BL/6 mice (age: 6-8 weeks) were administered with MCC950 (40 mg/kg) or saline vehicle through intraperitoneal injection. After 1 h, LPS (2 mg/kg) or saline vehicle was administered intravenously via tail vein. Epimedin B (40 mg/kg) was administered via an intraperitoneal injection after 2 h. Mouse serum and a fraction of liver tissues were collected after 6 h. H&E staining was performed, and the serum IL-1β, TNF-α, ALT, and AST levels were determined.

**Statistical analyses**

Prism 6 and SPSS statistics (version 21.0) were used for statistical analysis. All experimental data are expressed as mean ±SD. A two-tailed unpaired Student's t-test was conducted to evaluate significant differences between the two groups. Statistical significance was set at \( P < 0.05 \).

**Results**

**Epimedin B accelerates NLRP3 inflammasome activation**

In the present study, seven major active constituents of EF (epimedin A, epimedin A1, epimedin B, epimedin C, icariin, icaritin, and anhydroicaritin) were analyzed for their ability to activate the NLRP3 inflammasome. The results showed that only epimedin B significantly promoted the activation of caspase-1 and IL-1β production induced by nigericin in LPS-primed BMDMs (Fig. 1A-C) but did not affect the production of TNF-α (Fig. 1D). Therefore, only one major active constituent of EF could enhance nigericin-induced NLRP3 inflammasome activation.
To ascertain whether epimedin B could accelerate NLRP3 inflammasome activation, we examined the impact of epimedin B on caspase-1 activation and IL-1\(\beta\) secretion. Epimedin B exhibited dose-dependent active effects on caspase-1 cleavage, IL-1\(\beta\) secretion (Fig. 2A, B, D), and lactate dehydrogenase (LDH) induced by nigericin in LPS-primed BMDMs (Fig. 2C) but exerted no effect on inflammasome-independent cytokine TNF-\(\alpha\) production (Fig. 2E). We also assessed the effect of epimedin B on ATP-induced NLRP3 inflammasome activation. LPS-induced caspase-1 activation and IL-1\(\beta\) secretion in BMDMs could also be induced by epimedin B. However, it exerted no effect on TNF-\(\alpha\) production (Fig. 2F and G, Supplementary Fig. 1A-C). Additionally, we assessed the impact of epimedin B on nigericin-induced NLRP3 inflammasome activation in THP-1 cells. The results indicated that epimedin B enhanced caspase-1 maturation, IL-1\(\beta\) secretion, and LDH release in a dose-dependent manner in response to nigericin in PMA-primed THP-1 cells (Fig. 2H-K).

Pretreatment with epimedin B promoted nigericin-induced caspase-1 cleavage and IL-1\(\beta\) release in wild-type (WT) BMDMs but not in NLRP3-knockout (NLRP3-/-) BMDMs (Supplementary Fig. 3A). MCC950 is a small-molecule inhibitor of the NLRP3 inflammasome[24]. We further evaluated whether the activation of the NLRP3 inflammasome induced by epimedin B could be inhibited by MCC950. Our results indicated that epimedin B accelerated NLRP3 inflammasome activation. However, it could also be inhibited by MCC950 (Supplementary Fig. 3B).

Moreover, we explored the effect of epimedin B on NLRP3 inflammasome activation initiated in response to other stimuli. Unexpectedly, treatment with epimedin B exerted no effect on caspase-1 cleavage and IL-1\(\beta\) secretion stimulated by other NLRP3 agonists, including SiO2 and poly(I:C) (Fig. 3A, C-E). Epimedin B did not affect cytosolic LPS, NLRC4, or AIM2 inflammasome activation (Fig. 3B, F-H). Thus, these results indicate that epimedin B is a specific promoter that increases nigericin- and ATP-induced NLRP3 inflammasome activation.

We then examined whether epimedin B affected LPS-induced priming for inflammasome activation. When BMDMs were stimulated with epimedin B at doses of 10–40 \(\mu\)M before or after LPS treatment, epimedin B did not activate LPS-induced NLRP3 expression, IL-6, and TNF-\(\alpha\) production. Figure 4A-C suggests that epimedin B does not enhance LPS-induced priming at the doses that are effective for NLRP3 activation, suggesting that epimedin B exerts a robust effect on NLRP3 inflammasome activation.

**Epimedin B promotes nigericin or ATP-induced ASC oligomerization but does not block K\(^+\) efflux and Ca\(^{2+}\) flux**

We investigated the mechanism underlying the activation of NLRP3 by epimedin B. First, our studies showed that epimedin B could activate nigericin-induced ASC oligomerization (Fig. 4D), an essential step for NLRP3 activation, suggesting that epimedin B acted upstream of ASC oligomerization to exacerbate nigericin-induced NLRP3 activation. Second, epimedin B promoted ASC oligomerization induced by ATP (Fig. 4E). However, epimedin B demonstrated no impact on ASC oligomerization induced by SiO2, poly
Epimedin B facilitates NLRP3 inflammasome activation by increasing mitochondrial ROS production

Mitochondrial ROS play a crucial role in NLRP3 inflammasome activation[25]. Next, we studied whether epimedin B-mediated mitochondrial ROS was involved in NLRP3 inflammasome activation. Mitochondrial ROS production was not induced after epimedin B treatment alone. Epimedin B successfully potentiated mitochondrial reactive oxygen species production induced by nigericin and ATP but not by SiO2 (Fig. 5A-E). We focused on the ROS scavenger N-acetylcysteine (NAC), which is an inhibitor of mitochondrial ROS production. NAC was selected to evaluate whether the nigericin-dependent activity of epimedin B on NLRP3 activation was mediated by ROS mitochondrial production. Mitochondrial ROS production was suppressed by NAC treatment. As expected, when stimulated with nigericin, NAC treatment reversed epimedin B-induced caspase-1 maturation or IL-1β production (Fig. 5F-G). These results indicated that epimedin B increased mitochondrial ROS production to facilitate nigericin-induced NLRP3 inflammasome activation.

Epimedin B induces the development of IDILI by promoting NLRP3 inflammasome activation in vivo

We next examined whether epimedin B could induce the development of IDILI by promoting NLRP3 inflammasome activation in vivo. Co-exposure of animals to a non-hepatotoxic dose of LPS and drugs could mimic IDILI. First, we investigated whether epimedin B, which could activate the NLRP3 inflammasome, could also induce liver injury in an LPS-mediated susceptibility mouse model of IDILI. The results showed that treatment with epimedin B alone did not alter the levels of plasma ALT and AST compared with those of control mice. As expected, in the LPS-mediated mouse model, epimedin B increased the levels of ALT and AST and also induced an increase in the production of IL-1β and TNF-α compared to those in the LPS group (Fig. 6A-D). Similar results were observed in the mRNA expression of the pro-inflammatory genes IL-1β and IL-18 (Fig. 6E, F). Additionally, liver histology analysis showed that the combination of LPS and epimedin B treatment resulted in a trend of hepatocyte focal necrosis and
inflammatory cell infiltration in the liver tissue (Fig. 6G). To further explore the effects of epimedin B on the immunological reaction in the liver tissue, immunohistochemical (IHC) analysis of liver samples was performed. IHC staining of liver sections revealed that epimedin B increased the infiltration of F4/80-positive macrophages in the liver (Fig. 6H). Our results demonstrated that epimedin B could induce liver injury in the LPS-mediated susceptibility mouse model.

MCC950 is a potent selective NLRP3 inhibitor that is deemed a useful tool to mimic the consequences of NLRP3 inflammasome knockdown in mice[23]. To verify the relationship between the NLRP3 inflammasome and liver injury induced by epimedin B, mice treated with MCC950 were included. Figure 7 (A-D) indicates that the combination of LPS and epimedin B led to an increase in the levels of ALT, AST, IL-1β, and TNF-α but not in cotreated MCC950 mice. Moreover, mRNA expression of pro-inflammatory genes IL-1β, IL-18, and TNF-α was increased in the LPS group and the group subjected to treatment with LPS in combination with epimedin B but not in the MCC950 cotreatment group (Fig. 7E-G). As shown in Fig. 7H, MCC950 treatment suppressed caspase-1 activation in the liver tissues co-treated with epimedin B and LPS. Liver histology analysis showed that epimedin B induced hepatocyte focal necrosis and inflammation in the LPS-mediated mouse model but not in other groups (Fig. 7I). These results confirmed that epimedin B could activate the NLRP3 inflammasome, leading to liver injury in vivo.

Discussion

IDILI represents a major global health issue. For instance, in North America, it has already surpassed viral hepatitis as a major cause of acute liver failure[26, 27]. However, in recent years, IDILI caused by TCM has also been broadly recognized, especially in traditional non-toxic Chinese medicines. Thus, there is a lack of understanding of the possible IDILI mediated by TCM globally. Few traditional nontoxic Chinese medicines, such as Dictamni Cortex, Epimedii Folium, Psoraleae Fructus, and Polygoni Multiflori Radix have been reported to cause liver injury. Particularly, the Chinese Food and Drug Administration has reported that liver injury can be caused by two Chinese patent medicines, namely Zhuangguguanjiewan pills and Xianlinggubao capsules, which both contain Epimedii Folium. Furthermore, our previous studies have demonstrated that Epimedii Folium may induce hepatotoxicity in an LPS-mediated susceptibility mouse model of IDILI. Interestingly, in the present study, we demonstrated that epimedin B which is one of the constituents about EF could specifically reinforce the activation of the NLRP3 inflammasome, then to induce liver jury.

The NLRP3 inflammasome is one of the major contributors to inflammation and possesses the ability to sense both endogenous and exogenous danger signals through intracellular NLRs[12, 13]. NLRP3 inflammasome activation is also responsible for the development of several liver-related inflammatory diseases, especially chronic hepatitis C, nonalcoholic steatohepatitis, alcoholic liver disease, and IDILI[17, 28–33]. EF is a commonly used herbal medicine for promoting the functionality of liver and kidney. Our previous studies confirmed that EF could induce liver injury in an LPS-mediated susceptible mouse model of IDILI. However, the mechanisms underlying NLRP3 inflammasome-mediated hepatic inflammation of the major active constituents of EF remain elusive. In the present study, we demonstrated that epimedin
B, which is one of the major bioactive components, could facilitate nigericin- or ATP-induced NLRP3 inflammasome activation, leading to the development of IDILI. This suggests that epimedin B contributes to EF-induced idiosyncratic hepatotoxicity via enhancement of NLRP3 inflammasome activation.

In the present study, epimedin B could specifically reinforce the activation of the NLRP3 inflammasome induced by nigericin or ATP but not by SiO2, poly (I:C), and cytosolic LPS. Additionally, epimedin B exerted no effect on the activation of AIM2 and NLRC4 inflammasomes. These data indicate that epimedin B could specifically enhance nigericin- or ATP-induced NLRP3 inflammasome activation. We also examined the effects of epimedin B on upstream and downstream signaling, which is associated with NLRP3 inflammasome activation, and evaluated the mechanism underlying the enhancement of nigericin- or ATP-induced NLRP3 inflammasome activation by epimedin B. ASC oligomerization is a key event in NLRP3 inflammasome activation. Epimedin B promoted ASC oligomerization triggered by nigericin and ATP. Therefore, we noted that epimedin B acted on the upstream signaling events of ASC oligomerization to exacerbate nigericin- or ATP-induced NLRP3 inflammasome activation. K⁺ efflux is considered one of the main upstream events of NLRP3 inflammasome activation. However, our study indicated that epimedin B did not alter the K⁺ efflux triggered by nigericin. Ca²⁺ flux is also deemed one of the upstream mechanisms associated with NLRP3 inflammasome activation, but our results demonstrated that epimedin B did not alter Ca²⁺ flux triggered by ATP. Moreover, mitochondrial damage and the release of mitochondrial reactive oxygen species are key upstream events of NLRP3 inflammasome activation. This study showed that nigericin and ATP could induce the production of mitochondrial reactive oxygen species. Notably, epimedin B specifically amplifies the production of mitochondrial reactive oxygen species triggered by nigericin and ATP; but not by SiO2, suggesting that epimedin B may facilitate nigericin- or ATP-induced NLRP3 inflammasome activation dependent on mitochondrial ROS production. Next, we evaluated whether ROS played an important role in the enhanced effect of epimedin B on nigericin- or ATP-induced NLRP3 inflammasome activation. The results also indicated that NAC could inhibit NLRP3 inflammasome activation triggered by nigericin. We concluded that the effect of epimedin B was dependent on mitochondrial ROS production for facilitating nigericin-induced NLRP3 inflammasome activation.

We have previously reported that EF combined with non-hepatotoxic doses of LPS can induce liver injury. Some studies have also indicated that co-exposure to a non-hepatotoxic dose of LPS and drugs in animals could mimic IDILI. Therefore, we investigated whether epimedin B acting as an NLRP3 inflammasome activation promoter could cause liver injury in an LPS-mediated susceptible mouse model of IDILI. Our results indicated that epimedin B could induce liver injury in vivo in the LPS-mediated susceptibility mouse model. Thereafter, MCC950 was used to explore the relationship between liver injury induced by epimedin B and the NLRP3 inflammasome. Taken together, the combination of epimedin B and LPS induced liver injury but not in mice with MCC950 pretreatment. These data clearly demonstrated that epimedin B induced IDILI by promoting NLRP3 inflammasome activation in vivo.

**Conclusions**
In conclusion, our study demonstrated that epimedin B could induce NLRP3 inflammasome activation triggered by nigericin and ATP. Mitochondrial ROS are crucial contributors to the enhancement of the activation of the NLRP3 inflammasome stimulated by epimedin B. Treatment with the combination of nonhepatotoxic doses of LPS and epimedin B increased the production of ALT, AST, IL-1β, and TNF-α, resulting in hepatocyte necrosis. However, the results were not observed in mice co-treated with LPS and MCC950. Our study indicates that epimedin B, as a major risk component, is also responsible for EF-induced IDILI.

**Abbreviations**

IDILI: idiosyncratic drug-induced liver injury; EF: Epimedii Folium; HDS: herbal and dietary supplements; TCM: traditional Chinese medicine; NLR: the nucleotide-binding domain and leucine-rich repeat; PAMPs: pathogen-associated molecular patterns; IL-1β: interleukin 1β; BMDMs: Bone marrow-derived macrophages; DMEM: Dulbecco’s modified Eagle medium; FBS: fetal bovine serum; ATP: Adenosine triphosphate; DMSO: dimethyl sulfoxide; ELISA: Enzyme-linked immunosorbent assay; ALT: Alanine aminotransferase; AST: aspartate transaminase; LDH: Lactate dehydrogenase; ROS: reactive oxygen species.

**Declarations**

**Fundding**

This work was supported by the National Natural Science Foundation of China (82004057, 81874368) and the Beijing Nova Program (Z181100006218001).

**Acknowledgements**

None.

**Authors’ contributions**

Zhaofang Bai, Guang Xu, and Xiaohe Xiao supervised the project. Zhaofang Bai and Guang Xu designed the experiments. Yuan Gao and Wei Shi performed a considerable portion of the experiments. Can Tu performed mechanistic studies and analyzed the data. Guanyu Zhao analyzed the data derived from mice experiments. All authors read and approved the final manuscript.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**
All animal protocols in this study were performed according to the guidelines outlined for care and use of laboratory animals and approved by the Animal Ethics Committee of the Fifth Medical Centre, Chinese People’s Liberation Army General Hospital (animal ethics committee approval No. IACUC-2017-003).

**Consent for publication**

All co-authors agreed to publish the final version of the present manuscript.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures
Figure 1

Effect of the main constituents of Epimedii folium on NLRP3 inflammasome activation (A) Western blot analysis of the supernatants (Sup) and whole-cell lysates (Lys) derived from LPS-primed BMDMs subjected to treatment with the main constituents of EF (40 μM) and then stimulated with nigericin (10 μmol/L). (B-D) Caspase-1 activity (B) ELISA of IL-1β (C) and TNF-α (D) in Sup derived from the samples described in A. Data are expressed as mean ± SD from at least three biological samples. The significance
of the differences was analyzed using unpaired Student’s t-test: * P <0.05, ** P <0.01, *** P <0.001, NS; not significant, RLU; relative light units.

Figure 2

Epimedin B promotes NLRP3 inflammasome activation in BMDMs and THP1 cells stimulated by nigericin or ATP. (A) Western blot analysis of supernatants (Sup) and whole-cell lysates (Lys) derived from LPS-primed BMDMs subjected to treatment with various doses of epimedin B before nigericin stimulation. (B-
E) Caspase-1 activity (B), the release of LDH (C), ELISA of IL-1β (D) and TNF-α (E) levels in supernatants (Sup) from samples described in A. (F-G) Caspase-1 activity (F) and ELISA of IL-1β (G) of Sup and Lys derived from LPS-primed BMDMs subjected to treatment with various doses of epimedin B before ATP stimulation. (H) Western blots analysis of Sup and Lys derived from PMA-primed THP1 subjected to treatment with various doses of epimedin B before nigericin stimulation. (I-K) Caspase-1 activity (I), release of LDH (J), and ELISA of IL-1β(K) in Sup from samples described in H. Data are represented as mean ± SD from at least three biological samples. The significance of the differences was analyzed using unpaired Student’s t-test: *P <0.05, **P <0.01, ***P <0.001, NS; not significant, RLUs; the relative light units.
Epimedin B does not affect NLRP3 inflammasome activation induced by SiO2, poly (I:C), and intracellular LPS, as well as AIM2 and NLRC4 inflammasome. (A-B) Western blot analysis of supernatants (Sup) and whole-cell lysates (Lys) derived from LPS/Pam3CSK4-primed BMDMs subjected to treatment with epimedin B and then stimulated with nigericin, ATP, SiO2, poly (I:C), poly (dA:dT), Salmonella, or intracellular LPS. (C-H) Caspase-1 activity (C, F), ELISA of IL-1β (D, G), and TNF-α in Sup derived
Epimedin B promotes ATP or nigericin-induced ASC oligomerization but does not block K+ efflux and Ca2+ flux. (A) Western blot analysis of whole-cell lysates from BMDMs subjected to treatment with

**Figure 4**

Epimedin B promotes ATP or nigericin-induced ASC oligomerization but does not block K+ efflux and Ca2+ flux. (A) Western blot analysis of whole-cell lysates from BMDMs subjected to treatment with
epimedin B for 1 h; thereafter, they were stimulated with LPS (50 ng/mL) for 3 h or BMDMs were
stimulated with LPS (50 ng/mL) for 3 h and then subjected to treatment with epimedin B for 1 h. (B, C)
ELISA of TNF-α (B) and IL-6 (C) in Sup derived from samples described in A. (D) Western blot analysis of
ASC oligomerization from LPS-primed BMDMs subjected to treatment with various doses of epimedin B
before nigericin stimulation. (E) Western blot analysis of ASC oligomerization from LPS-primed BMDMs
subjected to treatment with epimedin B, following which they were stimulated with nigericin, ATP, SiO2,
and poly (I:C). (F) Quantification of potassium efflux in LPS-primed BMDMs subjected to treatment with
various doses of epimedin B and were then stimulated with nigericin. (G) A trace of ATP-induced Ca2+
flux was measured using the FLIPRTETRA system in LPS-primed BMDMs subjected to treatment with
epimedin B. Data are represented as mean ± SD from at least three biological samples. The significance
of the differences was analyzed using unpaired Student’s t-test: *P <0.05, **P < 0.01, ***P < 0.001, NS: not
significant.
Figure 5

Epimedin B facilitates NLRP3 inflammasome activation by increasing mitochondrial reactive oxygen species (mtROS) production (A-D) Percentage of ROS-positive cells in LPS-primed BMDMs subjected to treatment with epimedin B that were either not stimulated (A) or stimulated with nigericin (B), ATP (C) or SiO2 (D), followed by staining with MitoSox. After completion of the staining and washing procedures, flow cytometry was conducted to determine the level of mtROS production. (E) Percentage of ROS-
positive cells in LPS-primed BMDMs subjected to treatment with epimedin B that were either not stimulated or stimulated with nigericin, ATP, or SiO2. (F) Western blot analysis of supernatants and whole-cell lysates derived from LPS-primed BMDMs subjected to treatment with epimedin B, NAC, or epimedin B plus NAC before stimulation with nigericin or without stimulation. (G) Caspase-1 activity in samples described in F. Data are represented as mean ± SD derived from at least three biological samples. The significance of the differences was analyzed using unpaired Student's t-test: #P < 0.05, ##P < 0.01, ###P < 0.001, *P <0.05, **P <0.01, ***P <0.001, NS: not significant.
Figure 6

Epimedin B promotes early liver injury and inflammatory mediator production in vivo (A-H) Female C57BL/6 mice (age: 6-8 weeks) subjected to starvation for 24 h were administered with 2 mg/kg of LPS or its saline vehicle via the tail vein (i.v.). After an observation period of 2 h, various doses of epimedin B (20 mg/kg, 40 mg/kg, 80 mg/kg) or its vehicle were administered through intraperitoneal injection for 6 h. (A, B) Serum levels of ALT (A) and AST (B). (C, D) Serum levels of IL-1β (C) and TNF-α (D) determined by ELISA. (E, F) PCR of IL-1β (E) and IL-18 (F) mRNA levels. (G, H) Representative micrographs of H&E staining (G) and F4/80 staining (H). Scale bars represent 100 µm. Data are represented as mean ± SD. The significance of the differences was analyzed using unpaired Student’s t-test: *P <0.05, **P <0.01, ***P <0.001, NS: not significant.
Epimedin B induces IDILI by promoting NLRP3 inflammasome activation in vivo (A-I) Female C57BL/6 mice (age: 6-8 weeks) were administered with MCC950 (40 mg/kg) or its saline vehicle through intraperitoneal injection. After 1 h, LPS (2 mg/kg) or its saline vehicle was administered via the tail vein for 2 h. Subsequently, epimedin B (40 mg/kg) was administered via intraperitoneal injection for 6 h. (A, B) Serum levels of ALT (A) and AST (B). (C, D) Serum levels of IL-1β (C) and TNF-α (D) determined by ELISA.
(E-G) PCR of IL-1β (E), IL-18 (F), and TNF-α (G) mRNA levels. (H) Western blot analysis of pro-caspase-1 and cleaved caspase-1 expression in the liver tissue. (I) Representative micrographs of H&E staining. Scale bars represent 100 µm. Data are represented as mean ± SD. The significance of the differences was analyzed using unpaired Student's t-test: *P <0.05, **P <0.01, ***P <0.001, NS: not significant.

**Supplementary Files**

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