Original Research

Ursolic acid inhibits NLRP3 inflammasome activation and alleviates vascular smooth muscle injury in Kawasaki disease

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Abstract
Kawasaki disease (KD) is a kind of autoimmune disease with systemic vasculitis as the main pathological change. It is critical to explore potential new therapeutic agents to address KD disease and its complications. Ursolic acid (UA) is a pentacyclic triterpene (PT) carboxylic acid that has a number of important pharmacological activities, however, the possible effects of UA on the progression of KD and the mechanism are still unclear. Here we investigated the effects of UA on KD. We revealed that UA improved arterial injury in KD mice. UA improved vascular inflammation in KD mice. In addition, Ursolic suppressed NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome activation. We further found UA restrained vascular smooth muscle cell (VSMC) dedifferentiation, therefore suppressing KD progression. In summary, UA suppressed NLRP3 inflammasome activation as well as alleviated vascular smooth muscle injury in KD. We thought UA could act as a drug of KD.

Keywords
Kawasaki disease (KD); Ursolic acid (UA); Inflammation; NLRP3 inflammasome; Dedifferentiation

1. Introduction
Kawasaki disease (KD) is an autoimmune disease characterized by systemic vasculitis [1]. KD clinical features include persistent fever, bulbo-conjunctival congestion, changes in lips and mucous membranes, hand and foot dandruff, and rash [2]. Vasculitis involves small blood vessels, medium muscle arteries and organs across the body, especially coronary arteries [3, 4]. Coronary artery abnormalities (CAA), including aneurysms, myocardial fibrosis, and aortic root dilatation may result [5]. High-dose intravenous immunoglobulin (IVIG) have been found to be the most effective treatment for CAA, significantly reducing inflammation and CAA incidence [6]. However, up to 20% of KD patients remain resistant to IVIG and require adjuvant therapy [7, 8]. CAA still occurs in 5% of KD patients after IVIG treatment. Among children in developed countries, this is now the major cause of acquired heart disease and long-term cardiovascular complications [9]. Therefore, new therapeutic agents need to be explored to combat KD disease and its complications.

Ursolic acid (UA) is a pentacyclic triterpene (PT) carboxylic acid found in a variety of traditional herbs and foods, such as ginseng, apple peel, cranberries, plums, calendula, rosemary and pears [10]. To normal cells, UA and its derivatives are safe and low-toxic [11]. Further, several significant pharmacological activities make these compounds promising cancer treatment agents [12, 13]. UA and its derivatives have been shown to possess a variety of biological properties, including anti-inflammatory, antioxidant, neuroprotective and anti-diabetic effects, through various mechanisms [12–14]. The UA treatment prevents hypoxia/reperfusion (H/R)-induced cardiomyocyte apoptosis and mitochondrial dysfunction [15]. Liver fibrosis is reversed by UA through the inhibition of the NADPH Oxidase 4 (NOX4)/NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome activation. UA protects chondrocytes, exhibits anti-inflammatory properties and improves osteoarthritis by regulating the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB)/NLRP3 inflammasome pathway [17]. However, there is still confusion about the possible effects and mechanisms of UA on KD progression.

This study examined the effects of UA on KD and CAA. In the KD model, UA suppressed NLRP3 inflammasome activation and alleviated vascular smooth muscle damage. The UA could therefore be of use in treating KD.

2. Materials and methods

2.1 Animal treatment
The Kawasaki disease (KD) model was constructed by intraperitoneal injections of lactobacillus casei cell wall extract (LCWE, Sigma) at 500 µg into C57BL/6 male mice (n = 6 in each group) for 14 consecutive days. Grouping: (1) Control
group: normal saline injection. (2) UA group: intraperitoneal injection of UA (80 mg/kg, Sigma) for 14 days. (3) LCWE group: intraperitoneal injection of LCWE (500 µg) for 14 days. (4) LCWE + UA: intraperitoneal injection of LCWE (500 µg) and intragastrical administration of UA (80 mg/kg) for 14 days. Whole aorta tissues and blood were collected after the sacrifice.

2.2 Histological analysis
Artery tissues were fixed with 4% Paraformaldehyde (PFA), embedded in paraffin and sliced. Hematoxylin and eosin (H&E) were counterstained on the sections.

2.3 Enzyme-linked immunosorbent assay (ELISA)
Interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α and IL-6 levels in the serum were determined by ELISA kits following the manufacturer’s instructions. Samples were aspirated into wells. Biotin-conjugated primary antibodies were added before avidin conjugated Horseradish Peroxidase (HRP). Enzyme substrates were then added for color development. Using a microplate reader, we measured the intensity of each well.

2.4 Real-time PCR
Total RNA from arteries (frozen tissue in −80 °C) was extracted with TRIzol reagents (15596026, Invitrogen, Carlsbad, CA, USA). Reverse transcription of total RNA into cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) reverse transcriptase (Promega Corporation). The cDNA was amplified using the following primers: TNF-α: GGTTGCACTGTCTCAGCCTTT, GCCATAGAAGCTATGGGAGAG; IL-1β: ACAAGAGAAGAAGATGAC, GCTGTAGAGTGGACTTAT; IL-6: AGACAGCCACTCACC, TTCTGCCAGTGCCTCTT; Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH): AGAAGGCTGGGGCTCATTTG, AGGGGCCATCGAGAAGAAAGTAATGAC, GCTGTAGAGTGGGCTTAT; α-actin (ab8226, 1:3000, Abcam, Cambridge, UK). 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins and transfer them to polyvinylidene difluoride (PVDF) membranes. After incubation with 5% Bovine Serum Albumin (BSA), primary antibodies were used to target NLR Family Pyrin Domain Containing 3 (NLRP3) (ab263899, 1:1000, Abcam, Cambridge, UK), Apoptosis-associated Speck-like protein containing a CARD (ASC) (ab283684, 1:500, Abcam, Cambridge, UK), Caspase-1 (ab207082, 1:500, Abcam, Cambridge, UK), interleukin (IL)-1β (ab243091, 1:1000, Abcam, Cambridge, UK), interleukin (IL)-1β (ab254360, 1:500, Abcam, Cambridge, UK), and β-actin (ab8226, 1:3000, Abcam, Cambridge, UK). Tris-Buffered Saline with Tween 20 (TBST) rinsed membranes for 15 minutes, before incubating in 1:1000 HRP-conjugated secondary antibodies for 2 hours. An ECL detection kit was used to detect signals.

2.7 Statistics
GraphPad 8.0 software (GraphPad Software, San Diego, CA, USA) was used to analyze data. Student’s t test was conducted to analyze data among groups. Data were represented as mean ± standard deviation (SD). p < 0.05 was considered significant.

3. Results

3.1 Ursolic acid improves arterial injury in KD mice
LCWE-induced KD mice model was constructed to examine the role of UA in KD progression. A histological examination of aorta tissues was performed. LCWE induced mice exhibited progressive abdominal aortitis, immune cell infiltration, elastin destruction, necrosis, and medial thickening. These phenotypes were recovered after UA administration (80 mg/kg) (Fig. 1). Therefore, UA improved arterial injury in KD mice.

3.2 Ursolic acid improves vascular inflammation in KD mice
UA’s effects on aorta tissue inflammation were detected in the LCWE model. A lipopolysaccharide (LPS) model also monitors inflammatory cytokines. The mRNA levels of inflammatory factors, including TNF-α, IL-6 and IL-1β, were elevated in LCWE mice using qPCR assays (Fig. 2A). However, UA significantly rescued elevated levels of TNF-α, IL-6 and IL-1β in aorta tissues of LCWE mice (Fig. 2A). Aorta tissues of LCWE mice were also shown to secrete these inflammatory factors by ELISA assays, where UA rescued the inflammatory factors’ secretion (Fig. 2B). UA therefore reduced LCWE-induced release of inflammatory factors in KD mice.

3.3 Ursolic acid inhibits VSMC dedifferentiation
VSMC dedifferentiation was observed in aorta tissues from LCWE mice after UA treatment. We noticed that LCWE suppressed ACTA2 expression and increased PDGFRβ expression using immunofluorescence detection of PDGFRβ (dedifferen-
**FIGURE 1. Ursolic acid improves arterial injury in KD mice.** HE stainings showed mice’ aorta morphology in the Control, UA (80 mg/kg), LCWE and LCWE + UA groups. Scale bar indicates 100 μm. LCWE: lactobacillus casei cell wall extract; UA: Ursolic acid.

**FIGURE 2. Ursolic acid improves vascular inflammation in KD mice.** (A) qPCR assays showed mRNA levels of TNF-α, IL-6 and IL-1β in mice’ aorta tissues in Control, UA (80 mg/kg), LCWE and LCWE + UA groups. (B) ELISA assays showed the secretion of TNF-α, IL-6 and IL-1β in mice’ aorta tissues in the Control, UA (80 mg/kg), LCWE and LCWE + UA groups. ***p < 0.001. LCWE: lactobacillus casei cell wall extract; IL: interleukin; TNF: Tumor Necrosis Factor; UA: Ursolic acid; ns: no significant.
tiation marker) and ACTA2 (VSMC differentiation marker) in aorta tissues (Fig. 3). In contrast, UA treatment appeared to rescue LCWE-induced suppression of ACTA2 expression and promotion of PDGFRβ expression in aorta tissues (Fig. 3). So, UA suppresses VSMC dedifferentiation in KD mice.

3.4 Ursolic acid inhibits NLRP3 inflammasome activation

The role of UA in the NLRP3 pathway was examined to reveal the potential mechanism of UA-mediated effects in LCWE-induced mice. Immunoblot assays showed an increase in NLRP3 expression in the LCWE group, and UA treatment reversed it (Fig. 4A). Further, the LCWE-induced model activated the NLRP3 signaling pathway, as demonstrated by an increase in ASC, cleaved caspase-1, IL-1β and IL-18 (Fig. 4B). UA treatment inhibits ASC, cleaved caspase-1, IL-1β and IL-18 (Fig. 4B). It appears that UA suppresses LCWE-induced NLRP3 inflammasome activation.

4. Discussion

Kawasaki disease (KD) is a systemic vasculitis disease characterized by small, usually manifested by fever and rash [1]. Untreated, approximately 20% of children develop coronary artery damage. This may result in coronary artery dilation and coronary aneurysm formation, the most common causes of acquired heart disease [2, 18]. Despite the lack of clear etiology and pathogenesis, studies suggested that KD is related to bacteria, viruses, genetics and immunity [2]. TNF-α expression is upregulated during the acute phase of KD, which leads to vascular endothelial cell damage, barrier destruction, stimulates the expression of adhesion molecules and chemokines in the injured endothelial cells, and the recruitment of activated immune cells to the surface of damaged blood vessels, contributing to KD vascular injury [19]. Vasculitis begins with injury and dysfunction to vascular endothelial cells [20]. It is due to the expression and release of adhesion molecules by endothelial cells [20]. Our findings demonstrated that UA suppressed the secretion of inflammation factors, thus slowing KD progression.

Vasculitis is the main KD pathological process. Vascular smooth muscle cell VSMC dedifferentiation contributes to many vascular pathologies, including endothelial injury, thickening and aneurysm formation [20]. Assays on mice coronary artery smooth muscle cells using ELISA, qPCR and Immunoblot showed that UA improved vascular inflammation in KD mice, inhibited inflammasome activation and suppressed VSMC dedifferentiation. Thus, UA suppressed KD progression. Among its biological effects are sedation, anti-inflammatory, antibacterial, anti-diabetic, anti-ulcer and blood sugar lowering. According to studies, UA is anti-carcinogenic, induces F9 teratoma cell differentiation, and inhibits angiogenesis, serving as a potential anti-cancer drug with low toxicity and high efficiency [11–13]. UA also has an obvious antioxidant function [21]. The antioxidant properties of UA contribute to anti-aging and skin freckle removal [22]. UA inhibits 5-lipid oxidase and cycoperoxidase activity in the metabolism of arachidonic acid, preventing prostaglandins and leukotrienes formation, which may explain its inflammation and lipid peroxidation [23–25].

Caspase-1 mediated cell death is activated by seven types of inflammasomes, including NLRP3, NLRP1, NLRP6, NLRP9, AIM2, NLRC4 and Pyrin [26]. In particular, NLRP3 has been identified as a crucial NOD-like receptor protein that detect microbial and non-microbial danger signals and triggers sterile inflammatory responses [26, 27]. An activation of the NLRP3 inflammasome in coronary endothelial cells was found in a mice model of KD induced by Lactobacillus casei cell

FIGURE 3. Ursolic acid inhibits VSMC dedifferentiation. Immunostaining assays showed the expression and location of ACTA2 (red panel) and PDGFRβ (green panel) in mice’ aorta tissues in the Control, UA (80 mg/kg), LCWE and LCWE + UA groups. Scale bar indicates 100 µm. ***p < 0.001. LCWE: lactobacillus casei cell wall extract; ACTA: beta-actin, PDGFRβ: Platelet-Derived Growth Factor Receptor Beta; DAPI: 4′,6-diamidino-2-phenylindole; UA: Ursolic acid, ns: no significant.
F I G U R E 4. Ursolic acid inhibits NLRP3 inflammasome activation. (A) Immunoblot assays showed NLRP3 expression in mice’ aorta tissues in the Control, UA (80 mg/kg), LCWE and LCWE + UA groups. (B) Immunoblot assays showed the expression of ASC, cleaved caspase-1, IL-18 and IL-1β in mice’ aorta tissues in the Control, UA (80 mg/kg), LCWE and LCWE + UA groups. *p < 0.05, **p < 0.01, ***p < 0.001. LCWE: lactobacillus casei cell wall extract; IL: interleukin; NLRP: NLR Family Pyrin Domain Containing; ASC: Apoptosis-associated Speck-like protein containing a CARD; UA: Ursolic acid, ns: no significant.

wall extract. This led to a significant increase in caspase-1 activity and IL-1β production [28]. NLRP3 inflammasome regulates the development of LCWE-induced central vascular disease in KD in mice [28]. UA protects chondrocytes, exhibits anti-inflammatory properties and improves osteoarthritis by the NLRP3 inflammasome pathway [17]. Similarly, this study showed that UA suppressed the NLRP3 inflammasome activation in KD, thereby inhibiting its progression. However, further research is needed to determine the precise mechanism.

5. Conclusions

In summary, UA suppressed NLRP3 inflammasome activation and alleviated vascular smooth muscle injury in KD. Therefore, we consider UA a possible treatment for KD.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

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

AUTHOR CONTRIBUTIONS

GQC—performed material preparation and the experiments. YRW and YZ—performed data collection and analysis. TY and FG—wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors contributed to the study conception and design. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved for animal use by the Ethics Committee of Zhejiang Provincial People’s Hospital (Approval 20230517205613931071), and conducted according to the Guide for the Care and Use of Laboratory
Animals from the National Institutes of Health.

ACKNOWLEDGMENT
Not applicable.

FUNDING
This work was supported by the Zhejiang Medical Health Science and Technology Project (Grant No. 2023KY456), Zhejiang Medical Health Science and Technology Project (Grant No. 2021KY529) and Zhejiang Province traditional Chinese Medicine Science and technology Program (Grant No. 2022ZB039).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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How to cite this article: Guoqing Chen, Yanru Wang, Ying Zhang, Fang Gu, Tong Yu. Ursolic acid inhibits NLRP3 inflammasome activation and alleviates vascular smooth muscle injury in Kawasaki disease. Sigma Vitae. 2024; 204(4): 46-51. doi: 10.22514/sv.2024.041.