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Icariin alleviates murine lupus nephritis via inhibiting NF-κB activation pathway and NLRP3 inflammasome

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ABSTRACT

Aims: Lupus nephritis (LN) is a kidney inflammatory disease caused by systemic lupus erythematosus (SLE). Both NF-κB activation and NLRP3 inflammasome activation are implicated in LN pathogenesis, suggesting they are potential targets for LN treatment. Icariin, which is isolated from Chinese medicine Horny Goat Weed (Ying Yang Huo), has been shown to have anti-inflammation activity, and inhibit activations of both NF-κB and NLRP3 inflammasome. In present study, the effects of icariin on LN were evaluated in MRL/*lpr* mice.

Main methods: We treated MRL/*lpr* mice with icariin for 8 weeks and then analyzed the renal function and kidney pathology. We monitored the levels of anti-dsDNA antibody and the deposition of immune complex after icariin treatment. We also detected the macrophage infiltration, NF-κB activation, NLRP3 inflammasome activation and inflammatory cytokine TNF-α production in MRL/lpr mice after icariin treatment.

Key findings: We found that MRL/*lpr* mice treated with icariin displayed significantly attenuated the renal disease. Icariin-treated mice showed significantly reduced serum anti-dsDNA antibody level and immune complex deposition. Icariin inhibited NF-κB activation and TNF-α production in MRL/*lpr* mice. Icariin inhibited CCL2 production and macrophage infiltration in MRL/*lpr* mice. Finally, icariin suppressed NLRP3 inflammasome activation and IL-1β production in MRL/*lpr* mice.

Significance: Icariin alleviated murine lupus nephritis via inhibiting NF-κB activation and NLRP3 inflammasome activation.

1. Introduction

Systemic lupus erythematosus (SLE), also known as lupus, is an autoimmune disease characterized by autoantibodies production, immune complex deposition, leukocyte infiltration, complement activation and inflammatory cell-mediated tissue damage [1]. Lupus nephritis (LN), also known as SLE nephritis, is an inflammation of the kidneys caused by SLE. LN is one of the most serious complications of SLE, and a leading cause of morbidity and mortality in SLE patients. Although immunotherapy has improved the prognosis of SLE patients with renal disease considerably, a large proportion of patients with LN eventually progress to end-stage renal disease [2]. Therefore, elucidating the underlying mechanisms of LN and developing drugs to aim at corresponding targets for LN therapy are in urgent need [3].

It has been reported that nuclear factor-kappa B (NF-κB) signaling pathway is involved in LN pathogenesis [4]. NF-κB is a key regulator of the expression of numerous proteins involved in inflammation [5]. In steady state, NF-κB is sequestered by its inhibitor of NF-κB (IκB) in cytosol. Upon activation, IκB is phosphorylated by the IκB kinase (IKK) complex and then for degradation, which leads the nuclear translocation of NF-κB and initiation of downstream targets gene transcription including Tumor necrosis factor alpha (TNF-α), interlukine-6 (IL-6) [6]. Patients with LN have upregulated expression and activation of NF-κB in glomerular endothelial and mesangial cells, together with elevated inflammatory cytokines. The activation of NF-κB is well correlated with the LN severity, suggesting the essential role of NF-κB activation in LN pathogenesis [7]. Inhibitions of NF-κB activation have been shown to attenuate LN pathologies [3].

NLRP3 inflammasome has also been implicated in LN and increased expression of inflammasome components including NLRP3 and caspase-

Abbreviations: LN, Lupus nephritis; SLE, Systemic lupus erythematosus; Ying Yang Huo, Horny Goat Weed

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1 has been reported in LN [8]. Activation of NLRP3 inflammasome results in the activation of caspase-1 and release of mature IL-1β and IL-18. IL-1β plays essential role in LN pathogenesis and enhanced IL-1β is associated with LN [9]. It has been reported that in macrophages derived from lupus patients, the NLRP3 inflammasome activation was increased, which results in a feed-forward inflammatory loop contributes to disease flares and organ damage [10]. Therefore, NLRP3 inflammasome could be another potential therapy target of LN [3].

Icariin is a bioactive component isolated from Chinese medicine Horny Goat Weed (Ying Yang Huo). Diverse activities of icariin have been reported, including anti-inflammation [11], anti-atherosclerosis [12], anti-tumor [13]. It has also been well demonstrated that icariin inhibited both NF-κB and NLRP3 inflammasome activation [14–17]. For instance, it was found that icariin was able to decrease renal damage through inhibition of NLRP3 inflammasome activation in the rat model of nephropathy [17]. As mentioned above, NLRP3 inflammasome was involved in the pathology of LN, therefore, in current study, we explored the effects of icariin on LN. We demonstrated that icariin inhibited activations of both NF-κB and NLRP3 inflammasome, and attenuated the kidney pathology in *MRL/lpr* mice.

2. Materials and methods

2.1. Mice

MRL/lpr mice which developed the SLE-like phenotype were used in current experiments. Mice were purchased from Shanghai SLAC Laboratory Animal Company (Shanghai, China). Wild type C57BL/6 mice with matched sex and age were used as control. The use of animals was followed with National Institutes of Health Guide for Care and Use of Animals and approved by The First Affiliated Hospital of Wenzhou Medical University. Mice were maintained in a constant temperature (23 °C) and 14 h light/10 h dark cycle environment and provided with ad libitum feeding of water and food.

2.2. Experimental design

Ten weeks old MRL/lpr mice were divided into two groups randomly, with 10 mice in each group. Icariin was purchased from the National Institute for the control of Pharmaceutical and Biological Products (Beijing, China), and was dissolved in saline with ultra-sonication. Group1 was considered as vehicle control group and given saline (vehicle group). Group 2 was the icariin treated group and administrated with icariin (10 mg/kg/day) by gavage every day for total 8 weeks as described before [17]. Wild type C57BL/6 mice were used as non-treated group (control group).

2.3. Renal function evaluation

Urine samples were collected every two weeks in metabolic cages with free access to water and standard diet. Urine protein was semi-quantified by Multistix 10SG reagent strips and analyzed by Clinitek Sttus analyzer (Bayer Healthcare), and graded on a scale of 0–4, 0 = non, 1 = 30–100 mg/dl, 2 = 100–300 mg/dl, 3 = 300–2000 mg/dl, or 4 > 2000 mg/dl, as described previously [18]. Blood were obtained from the eyeball and then centrifuged at 3000 rpm for 10 min to separate the serum for further analysis. Serum creatinine (Scr) and blood urea nitrogen (BUN) levels were measured using a Cobas®C311 Autoanalyzer (Roche Diagnostics, Indianapolis, USA).

2.4. Histology analysis

For pathology and immunohistochemistry, 10% formalin-fixed and paraffin-embedded renal tissues were cut into 2 μm thickness. After deparaffinization, the slides were stained with hematoxylin (HE) reagents for pathologic evaluation as described previously [19].

2.5. Immunofluorescence of immune complex deposition

To evaluate the renal immune complex deposition, frozen kidney sections were stained for mouse immunoglobulins with FITC-conjugated rabbit anti-mouse IgG (Santa Cruz, Dallas, USA) after blocking with 10% fetal bovine serum. The mean intensity of green fluorescence was scored as 0–3 as previously described. For each mouse, 50 glomeruli were analyzed and the sum of each glomeruli mean was used for final analysis.

2.6. ELISA

The serum level of anti-DNA IgG antibody was determined by ELISA. Briefly, 96-well ELISA plates were coated with 5 μg/ml calf thymus dsDNA (Sigma-Aldrich, St. Louis, USA). After blocking with 1% BSA, sera were added and incubated at room temperature for 1 h. Normal mouse IgG was used as negative control. Standard curve was prepared by using mouse anti-dsDNA monoclonal antibody (Abcam, Shanghai, China). After washing, peroxidase conjugated goat anti-mouse IgG antibody (Sigma, USA) was added to detect the bound anti-dsDNA antibody followed by the peroxidase substrate. The absorbance was measured at 450 nm. The concentration of anti-dsDNA was calculated using the standard curve.

Commercial ELISA kits for TNF-α and IL-1β were purchased from R &D systems (Minneapolis, MN, USA) and used for cytokines measurement in kidney homogenates and serum according to manufacturer's instructions.

2.7. Western blot

Total kidney proteins were extracted using cell lysis buffer (Cell Signaling Technology, USA) according to manufacturer's instruction. In some experiment, NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher, USA) were used to isolate nuclear and cytosolic proteins according to manufacturer's instructions. A total of 40 μg of proteins were loaded onto a 12% SDS-PAGE gel. After transfer, membranes were blocked by 5% non-fat milk and incubated with different primary antibodies: Anti-p-IκB (1:1000, Cell Signaling technology, Danvers, MA, USA), Anti-NFκB (1:1000, Santa Cruz, Dallas, TX, USA), Anti-F4/80 (1:1000, Abcam, Cambridge, MA, USA), Anti-CCL2 (1:1000, Abcam, USA), Anti-Capase-1p20 (1:1000, Santa Cruz, USA), Anti-β actin (1:1000, Sigma, USA), Anti-fibrillarin (1:500, Sigma, USA), and anti-caspase-1 p20 (1:1000, Santa Cruz, USA), for overnight at 4 °C. Next day, corresponding HRP-conjugated secondary antibodies were incubated. Peroxidase reaction was visualized by the SuperSignal West Pico Chemiluminenscent Substrate Kit (Thermo Scientific, USA). The bands intensities were quantitated using the Image J software.

2.8. Statistical analysis

Data were presented as mean ± SD. One-way ANOVA analysis and Tukey's post-hoc test were used. Statistical difference was considered as significant only if $p < 0.05$.

3. Results

3.1. Icariin attenuated renal lesions in MRL/lpr mice

To determine the potential effects of icariin on renal function in MRL/lpr mice, 10 weeks MRL/*lpr* mice were administrated icariin for 8 weeks and then analyzed for renal function and pathology. As shown in Fig. 1A, in vehicle treated MRL/*lpr* mice, urine protein scores increased with time increased, which indicated attenuated renal function in MRL/*lpr* mice. In contrast, MRL/*lpr* mice treated with icariin had unchanged urine protein scores and the scores on 16 and 18 weeks were significantly lower than the scores in vehicle-treated mice. Thus, this