An active fraction from Polyrhachis vicina Roger (AFPR) suppressed uric acid-induced inflammation through inhibition of NF-κB signaling in vivo and in vitro

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INTRODUCTION

Uric acid (UA) is the end product of purine metabolism and excreted in the urine. Epidemiologic and experimental evidence indicated that UA is an independent risk factor for hypertension, cardiovascular, obesity, dysglycemic conditions, diabetes mellitus and organ damage1-4. One possible explanation for how UA might contribute to organ damage is that UA might possess the ability to induce inflammation5,6. Increasing evidence demonstrated that the tissue excessive accumulation of UA lead to gouty inflammation in organs, and thus resulted in organ damage8,9.

ABSTRACT

Objective: To investigate the anti-inflammatory effect of active fraction of Polyrhachis vicina Roger (AFPR) in hyperuricemic rat and in uric acid (UA)-induced CLONE9 liver cells, and to elucidate the underlying mechanism. Methods: Uric acid (UA) -induced CLONE9 cells and hyperuricemic rat model were used to assess the anti-inflammatory effect of AFPR. Both mRNA and protein expressions of iNOS, COX-2, NF-κB and pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) in UA -induced CLONE9 cells were evaluated by qRT-PCR and Western blot method. Effect of AFPR on NF-κB signaling in UA -induced CLONE9 cells was demonstrated by overexpression of NF-κB. Serum levels and mRNA levels of IL-6, IL-1β, TNF-α, CRP and C3 in the liver tissues of hyperuricemic rat were detected by ELISA and qRT-PCR method. Results: We demonstrated that both mRNA and protein expressions of iNOS, COX-2, NF-κB and pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) were upregulated in UA-stimulated CLONE9 cells, while AFPR treatment could attenuate these inflammatory responses. AFPR significantly decreased mRNA levels and serum levels of IL-6, IL-1β, TNF-α in the liver tissues of hyperuricemic rat. Furthermore, the western blot analysis showed that AFPR exerted its anti-inflammatory effects mainly through the inactivation of NF-κB signaling pathway. Conclusions: Our studies suggested AFPR exerted anti-inflammatory effect through inhibition of NF-κB signaling in hyperuricemic rat and UA-induced CLONE9 cells. These results shed light on the potential therapeutic effects of AFPR on hyperuricemia, gouty inflammation and gout-induced organ damage.

Keywords: Polyrhachis vicina Roger, uric acid, inflammation, NF-κB

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There is evidence that UA can induce inflammatory reactions in human vascular cells, murine kidney tubular cells,
and pancreatic β-cells of mice treated for 4 weeks with daily intraperitoneal injections of UA. A study by Yang Zhou et al. reported that UA induced renal inflammation by recruiting T cells and macrophage infiltration and pro-inflammatory cytokines and chemokines expression in hyperuricemia mice kidneys. Significantly, UA interacted with pathogen-recognition receptors and led to nuclear factor-kB (NF-kB) activation and formation of a protein complex called NLRP3 inflammasome, resulting in the activation of caspase-1 and processing and secretion of IL-1β, a pro-inflammatory cytokine. IL-1β along with other pro-inflammatory cytokines, TNF-α, IL-6, and IL-8, promoted neutrophil influx, the primary pathological hallmark of gout. The development of therapeutics targeted to specific pro-inflammatory signal-transduction cascades and cytokines potentially applicable to the gout-related diseases treatment is rapidly advancing. However, the management of gout remains suboptimal due to the undesirable side effects such as gastrointestinal toxicity, bleeding, diarrhoea, and cardiovascular events. As a result, there is an urgent need to develop new safe anti-inflammatory treatments with maximum efficacy for gouty inflammation therapy.

Polyrhachis vicina Roger, a traditional Chinese medicine, is proved to possess good anti-depressant, anti-tumor and immune-regulation activities. Recent studies from our laboratory found that an active fraction from Polyrhachis vicina Roger (AFPR) could significantly suppressed the hyperuricemia in vivo, accompanied by a down-regulation of IL-6, IL-1β and TNF-α expression. Up to now, no report has been issued on its anti-inflammatory activities and prevented gout-induced organ damage. Therefore, as a part of our ongoing screening program to evaluate the anti-inflammatory properties of AFPR, we investigated the suppressive effect of AFPR and its underlying molecular mechanisms against inflammatory immune responses.

METHODS AND MATERIALS

Chemicals and Reagents

Potassium oxonate and allopurinol were purchased from Sigma-Aldrich Chemical Company. Commercial kits for CRP, α1-antitrypsin, TNF-α, IL-6, and C3 were purchased from R&D Systems (Minneapolis, USA). Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin/mL and 100 ng of streptomycin/mL. CLONE 9 cells were maintained in a 37°C incubator with 5% CO₂.

Preparation of AFPR

Dried Polyrhachis vicina Rogers were provided by a local vendor of Chinese materiamedica in Nanning city (Guangxi, China). The voucher specimen (PR-201505) was identified by Professor Xianbiao Zeng from Guangxi Institute of Chinese Medicine & Pharmaceutical Science and was deposited in the Herbarium of Guangxi Institute of Chinese Medicine & Pharmaceutical Science. Dried Polyrhachis vicina Rogers (1000 g) were milled and extracted 3 times with 10 L of ethanol/water (95:5, v/v) in a reflux system (each extraction for 1 h). The extracts were merged and filtered, followed by a water bath at 75±5 °C to obtain a crude aqueous ethanol extract (CAE). CAE was extracted 3 times by petroleum ether. The extracts were merged and filtered, followed by a water bath at 75±5 °C to obtain the active fraction (AFPR), with the yielded weight of 3.96% of dried Polyrhachis vicina Rogers (w/w). GC-MS analysis showed that unsaturated fatty acids comprise 71.14% of the major components, with 60.77% of octadecenoic acid, 9.31% of heptadecenoic acid and 1.06% of linoleic acid, saturated fatty comprise 25.04% of the components in AFPR. All other reagents were standard laboratory reagents of analytical grade.

Animals

Adult male Sprague-Dawley rats (200-250 g) were supplied by the Center of Experimental Animal of Guangdong Pharmaceutical University and were housed (5 per cage) under standard conditions (12-h light/dark cycle; 22±1°C ambient temperature; and 55±10% relative humidity), with free access to food and water. All experimental procedures were in accordance with the National Institutes of Health for the care and use of laboratory animals and were granted approval from the Animal Care Committee of Guangxi Institute of Chinese Medicine & Pharmaceutical Science (Approval Number GICMP-2018-0515).

Cell Culture

Rat liver cell lines CLONE9 was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin/mL and 100 ng of streptomycin/mL. CLONE 9 cells were maintained in a 37°C incubator with 5% CO₂.

UA-induced Inflammation in CLONE9 Cells

CLONE9 cells (rat liver cell lines) in the logarithmic phase of growth were seeded at 3,000/well and cultured in 96-well plates. To assess the effect of AFPR on inflammatory responses induced by UA, CLONE9 cells were incubated with UA (4μg/mL) for 24 h, and then treated with AFPR (6 mg/mL) for another 24 h.

Cambrian, et al.: AFPR suppressed UA-induced inflammation

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Expressions of iNOS, COX-2, NF-κB, IL-6, TNF-α and IL-1β in UA-induced CLONE9 Cells by Quantitative Real-time PCR Method

Total RNA was isolated from cultured cells mentioned above with TRIzol reagent according to the manufacturer’s instruction (Invitrogen). To determine the quantity of the mRNA levels, total RNAs were reverse transcribed by oligodeoxynucleoside primer using the PrimeScript RT Reagent Kit (Vazyme Biotech co.,ltd, China). The housekeeping gene GAPDH was used as an internal control. The primers were as follows: GAPDH forward primer, 5′-GACAGCCGCATCTTCCTTG-3′; GAPDH reverse primer, 5′-GAGAAAGCAGCCCTTGTAAC-3′; IL6 forward primer, 5′-ATTGTATGAAACGCAGTATGC-3′; IL6 reverse primer, 5′-CCAGGTAGAACCAGAACTCCAGA-3′; I L I B f o r w a r d p r i m e r , 5′-CCCTGAACACTCTGGAATAGCA-3′; IL1B reverse primer, 5′-CCCAAGTCAAGGGCTTGGA-3′; TNF forward primer, 5′-AGACATCTTCAGCCCTTGTTAGG-3′; TNF reverse primer, 5′-ACCTTGAGAATTGCTCAG-3′; iNOS forward primer, 5′-TCTTTGAGCCTGCGACTGTAGA-3′; iNOS reverse primer, 5′-CGTGAAAGCAGCATTTTGGCT-3′; COX2 forward primer, 5′-ATCTGGCTTCGGAACACAC-3′; COX2 reverse primer, 5′-GAGGCCATTGCTTCTGTGATCTG-3′; N F K B I f o r w a r d p r i m e r , 5′-GGAGGCCATGTTCGTTAGG-3′; NFKB1 reverse primer, 5′-CCCTGCGTGTGGATTTC-3′; Quantitative RT-PCR was performed using SYBR Premix Dimer Eraser (Vazyme Biotech co.,ltd, China) on a 7900HT system (Applied Biosystems, USA). GAPDH levels were used as an internal control, and fold changes were calculated by relative quantification (2^ΔΔCt).

Expressions of IL-1β, IL-6, TNF-α, pNF-κB p65, pI kBα in UA-induced CLONE9 Cells by Western Blotting Method

Cells grounded in liquid nitrogen were lysed on ice for 30 min in RIPA buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM aprotinin, 2 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 mM peptatin A. The lysates were centrifugated at 12,000 rpm at 4°C for 15 min, the supernatants were collected, and protein concentrations were determined using bicinchoninic acid assay. Protein extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes in transfer buffer (20 mMTris, 150 mM glycine, 20% [volume/volume] methanol). Membranes were blocked with 5% nonfat dry milk for 2 h and incubated with primary antibodies (IL-1β, IL-6, TNF-α, pNF-κB p65, pI kBα: Proteintech, China; β-actin: Abcam, USA). The protein bands were probed with secondary antibody, and visualized with the electrochemiluminescence detection system (Thermo Scientific, USA).

Effects of AFPR on NF-κB Signaling in UA-induced CLONE9 Cells

To confirm whether the inhibitory effects of AFPR on UA-stimulated inflammatory mediators are due to its influence on the activation of NF-κB signaling, we transfected Nfkb1 plasmid into CLONE9 cells for 6 h with AFPR (6 mg/ml) pre-treated for 24 h prior to the stimulation of UA. Then protein expressions of p-NF-κB p65, p-IκBα, IL-6, IL-1β and TNF-α were evaluated by western blotting method mentioned above.

Establishment of Hyperuricemic Rat Model and Drug Administration

In the present study, an experimental model of hyperuricemia induced by potassium oxonate was adopted according to Lemos Lima Rde C et al.22 with modifications. Fifty male Sprague-Dawley rats were randomly divided into 5 groups: control, model, allopurinol(20 mg/kg), AFPR(3.65mg/kg), AFPR(15.60mg/kg) (n=10). Apart from control group, other rats were i.g administered with potassium oxonate (650 mg/kg, ig) once daily for 11 weeks to induce hyperuricemia. During 11 weeks, rats from drug-treated groups were respectively administered with AFPR or allopurinol at different dosages. Rats from control group and model group were given equivalent water. Allopurinol group was designed as positive control.

Blood Sample Collection

On the 77th day (the last day of the 11th week), 1 h after drug administration, blood samples were collected through the retro orbital plexus region. Then serum was obtained by centrifugation at 3500×g for 5 min.

Determination of UA in Serum

To assess establishment of the hyperuricemia model and therapeutic effects of AFPR, serum UA content at 11th week was measured with phosphotungstic acid method23, according to the manufacturer’s instruction (Jiancheng Institute of Biotechnology, Nanjing, China).

Measurement of Pro-inflammatory Cytokines in Serum and Liver

Serum levels of IL-6, IL-1β, TNF-α, CRP and C3 were measured using ELISA kits, respectively, according to manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). Total RNA from liver tissue was extracted with TRIzol reagent according to the manufacturer’s instruction (Invitrogen). Then mRNA levels of IL-6, IL-1β, TNF-α in liver tissue were detected by quantitative real-time PCR method mentioned above.
Statistical Analysis

Results were expressed as mean ± SD and statistical analysis was performed by SPSS 16.0 software, using one-way ANOVA to determine the significant differences between groups, followed by Student’s Newman-Keul’s test. \( P<0.05 \) was considered statistically significant.

RESULTS

AFPR Attenuate the Inflammatory Responses Induced by UA in CLONE9 Cells

UA has been proven to be associated with inflammatory biomarkers. Our previous study found that AFPR reduced the serum levels of UA in hyperuricemia rats. To assess the effect of AFPR on inflammatory responses induced by UA, CLONE9 cells, rat liver cell lines, were incubated with UA (4 μg/ml) for 24 h, and then treated with AFPR (6 mg/mL) for another 24 h. The expressions of IL-6, TNF-α, IL-1β, iNOS, COX-2 and NF-κB were examined by qRT-PCR and Western blot method. We found that AFPR treatment significantly decreased both the mRNA and protein levels of IL-6, TNF-α, IL-1β, iNOS and COX-2 and NF-κB in UA-stimulated CLONE9 cells (Figure 1A-H). These findings demonstrated that AFPR could attenuate the inflammatory responses caused by UA induction, suggesting that AFPR has the potential to be further developed into an anti UA-induced inflammation drug.

Effect of AFPR on UA-induced NF-κB Signaling Pathway in CLONE9 Cells

NF-κB signaling is important in inflammation, and IL-6, TNF-α, and IL-1β have been shown to be regulated by NF-κB signaling. As showed in Figure 2A-B, the western blot analysis clearly showed that AFPR mainly exerts its anti-
inflammatory effects by inhibiting the UA-induced p-IκBα and p-NF-κB p65 protein expression in CLONE9 cells, which subsequently reduced the expressions of IL-6, IL-1β and TNF-α protein. Interestingly, NF-κB over-expression by Nfkb1 plasmid could partly rescue the inhibition of p-NF-κB p65, p-IκBα, IL-6, IL-1β and TNF-α by AFPR, suggesting that AFPR suppressed UA-induced inflammation via NF-κB signaling pathway.

Figure 2: Effect of AFPR on UA-induced NF-κB signaling pathway in CLONE9 cells. (A-B) Protein expression of cytokines by Western blot assay. △P < 0.05, △△P < 0.01 vs. UA- treated group. *P < 0.05, **P < 0.01 vs. Control group. 

Figure 3: Effect of AFPR against inflammatory responses in hyperuricemia rats. (A) Serum UA level o in hyperuricemia rats.(B) Serum IL-6 level o in hyperuricemia rats.(C) Serum IL-1β level o in hyperuricemia rats. (D) Serum CRP level o in hyperuricemia rats. (E) Serum TNF-α level o in hyperuricemia rats. (F) Serum C3 level o in hyperuricemia rats. (G) mRNA levels of cytokines by qRT-PCR assay. △P < 0.05, △△P < 0.01 vs. model group. *P < 0.05, **P < 0.01 vs. control group
AFPR Suppressed Inflammatory Responses in Hyperuricemia Rats

Previous study from our laboratory confirmed that AFPR had promising anti-hyperuricemia effect against hyperuricemia in rats. In order to explore extensive pharmacological action of AFPR in hyperuricemia rats, we established an experimental model of hyperuricemia induced by potassium oxonate in rats and then treated the models with AFPR or allopurinol (positive control). On the last day of the 11th week, blood samples were collected, and the serum levels of UA, IL-6, IL-1β, TNF-α, CRP and C3 were detected. The results showed that the serum UA content in hyperuricemia model group was significantly increased compared to control group, and AFPR remarkably inhibited UA levels when compared to the model group (Fig.3A). Furthermore, ELISA assays showed that the changes of IL-6, IL-1β, TNF-α, CRP and C3 levels were consistent with UA (Figure.3B-F). qRT-PCR assays showed that the mRNA levels of IL-6, IL-1β, TNF-α in the liver tissues of the model group were increased compared with that of normal group, while those of the AFPR groups were decreased compared with that of the model group (Figure.3G). As demonstrated in UA-induced CLONE9 cells, UA might activate NF-κB signaling pathway, resulting in inflammation response. In vivo study results suggested that AFPR reduced serum UA levels and subsequently suppressed UA-induced inflammatory responses in hyperuricemia rats.

DISCUSSION

Increasing evidences were emerging to support the conclusion that inflammation is the central mechanism for liver and kidney injury in hyperuricemic rodents and patients. In addition, Several lines of evidence indicate that excessive UA accumulation is one of the most potent pro-inflammatory stimuli which can initiate, amplify, and sustain an intense inflammatory response in the joint cavity and organs, which leads to gouty arthritis and organ inflammatory injury. UA could activate the formation of inflammasome, a mutimeric protein complex, which in turn cleaves pro-IL-1β to active IL-1β by activating caspase-1. IL-1β is the pivotal inflammatory mediator that regulates cell proliferation, differentiation, and apoptosis in gouty arthritis. In addition, IL-1β induce the expression of a wide range of cytokines and chemokines like TNF-α, IL-6, IL-17, and MCP-1. UA might activate the inflammatory mediator that regulates cell proliferation, differentiation, and apoptosis in gouty arthritis. In order to explore the AFPR anti-inflammatory molecular mechanisms against hyperuricemia, we investigated the inhibitory effect of AFPR on the expression levels of pro-inflammatory cytokines and classical inflammatory regulatory factors in UA-induced rat liver cells CLONE9, an experimental in vitro model for acute high uric acid in liver cells. Our research showed that AFPR remarkably decreased the mRNA and protein expressions of the inflammatory regulatory factors and the pro-inflammatory cytokines, including iNOS, COX-2, NF-κB, IL-6, IL-1β and TNF-α, in UA stimulated CLONE9 cells. It suggested that AFPR attenuated the UA induced inflammatory responses through inhibiting the production of pro-inflammatory mediators.

NF-κB signaling, a principal signaling pathway mediating pro-inflammatory cytokine (TNF-α, IL-1β, IL-6 and IL-17) genes involved in immune responses in various conditions, was activated in UA-induced cells. The classical pathway leading to the activation of NF-κB involved an IκB kinase (NF-κB inhibitor) complex. On activation, the IKK complex phosphorylated IκB leading to its degradation. This enabled the active NF-κB transcription factor subunits such as p65 to translocate to the nucleus and induce target gene expression. Thus, the drugs that have the capability to inhibit the phosphorylation of NF-κB p65 could block the nuclear translocation of NF-κB. In our research, we found that NF-κB over-expression could partly rescue the inhibition by AFPR on the expression of p-IκBz,p-NF-κB, IL-6, IL-1β and TNF-α protein levels in UA stimulated liver cells. These results demonstrated that AFPR suppressed the expression of inflammatory mediators (IL-6, IL-1β and TNF-α) through the inactivation of NF-κB signaling pathway in UA stimulated liver cells.

In fact, the serum UA level is a representative biomarker of hyperuricemia. The mRNA and protein levels of IL-6, IL-1β, TNF-α, CRP and C3 were upregulated in hyperuricemia model group, while suppressed in AFPR high dose group. Our findings suggested that the anti-hyperuricemic effects of AFPR might be due to its anti-inflammatory bioactivity.

In conclusion, we confirmed the anti-inflammatory activities of AFPR in UA induced CLONE9 cells. We provided evidence that AFPR treatment could significantly suppressed the pro-inflammatory cytokine expression, which was underlain by the mechanism of the inhibition of NF-κB signaling activation. Our study may help understand the anti inflammatory mechanism of AFPR against hyperuricemia and shed light on the potential therapeutic application of AFPR on hyperuricemia, gouty-inflammation, and gout-induced organ damage.

REFERENCES


