The protective effects of S14G-humanin (HNG) against mono-sodium urate (MSU) crystals- induced gouty arthritis

Jihui Zhang, Hongwei Lei, and Xiu Li

Department of Rheumatism and Immunology, The Second Affiliated Hospital of Harbin Medical University, Harbin City, Heilongjiang Province, China

ABSTRACT
Gout is a common and complex form of arthritis that has brought great inconveniences to the normal lives of patients. It is reported that oxidative stress and nod-like receptor family protein 3 (NLRP3) inflammasome-mediated inflammatory reactions are involved in the pathogenesis of gout arthritis. S14G-humanin (S14G-HNG) is a modified peptide of HNG with higher inhibitory activity on the accumulation and deposition of Aβ. Recently, S14G-HNG has been reported to exert great anti-inflammatory effects. The present study proposed to explore the possible therapeutic property of S14G-HNG against gout arthritis. An animal model was established by stimulation with mono-sodium urate (MSU) crystals, followed by treatment with colchicine and S14G-HNG, respectively. The elevated Gait score promoted synovitis score and activated myeloperoxidase (MPO) observed in MSU crystals-treated mice were significantly reversed by colchicine and S14G-HNG. Bone marrow-derived macrophages (BMDMs) were isolated from mice and stimulated with MSU crystals, followed by being treated with 25 and 50 μM S14G-HNG. The increased mitochondrial reactive oxygen species (ROS) and Malondialdehyde (MDA) levels, upregulated NADPH oxidase-4 (NOX-4), activated NLRP3 inflammasome, and elevated production of inflammatory factors in MSU crystals-treated BMDMs were dramatically reversed by S14G-HNG, accompanied by the upregulation of sirtuin type-1 (SIRT1). Lastly, the protective effects of S14G-HNG against MSU crystals-induced NLRP3 inflammasome activation were significantly abolished by the knockdown of SIRT1. In conclusion, our data reveal that S14G-HNG could possess potential benefits against MSU crystals-induced gout arthritis, with colchicine displaying a better effect.

Introduction

The morbidity of gout increases annually as diet and lifestyle develop and change over the years. Epidemiological data indicate that the global prevalence rate of gout is 1.4–3.9%, and the male/female ratio is 2.9:3.6 [1,2]. In China, the prevalence rate of gout is 0.15–1.14% [3], and it has become a great threat to public health [4]. Investigations on the pathological mechanism have led to more recognition of the pathogenesis of gout. Elevated blood uric acid is induced by purine metabolism disorder and reduced uric acid excretion, which leads to the excessive accumulation of monosodium urate (MSU) crystals in joints to trigger the breakout of acute gouty arthritis [5]. Chronic gouty arthritis is ultimately induced by repeated acute gouty arthritis attacks, which is a common pathological progression observed in gout patients [6]. During this progression, located macrophages play an important role in initiating the inflammation [7]. Martin [8] reported that under the stimulation of MSU crystals, the production of inflammatory factors precedes the infiltration of monocytes and neutrophils. In addition, the secretion of inflammatory factors and infiltration of neutrophils could be suppressed by removing the located macrophages. Therefore, in the early stage of gout, macrophages are the main immune cells responsible for the development of inflammation, but not monocytes and neutrophils. MSU crystals activate located macrophages through the Toll-like receptors (TLRs) and NLRP3 inflammasome pathway to stimulate the transcription of the interleukin-1β (IL-1β) precursor [9]. In animal experiments, Martin [10] reported that after stimulation with MSU crystals, M1 polarization of infiltrated macrophages is induced, and multiple types of...
inflammatory cytokines, such as IL-1β, tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein (MCP)-2, and neutrophil chemotactic factors (NCFs), are released by the M1 macrophages, contributing to the progression of gout arthritis. Several inflammatory factors are involved in the pathogenesis of gout arthritis, such as IL-1β, IL-6, IL-18, TNF-α, and IL-8. IL-1β is regarded as the key factor involved in the inflammation mediated by MSU crystals. The IL-1β precursor is produced by macrophages and dendritic cells, and is processed to IL-1β by the NLRP3 inflammasome activating protease-1 to mediate the inflammatory reaction [11,12]. IL-18 is another inflammatory factor mediated by the NLRP3 inflammasome. It mainly originates from cartilage, synovial cells and endothelial cells in gout arthritis patients, and accelerates the inflammatory reaction by inducing the production of interferons [12]. Therefore, NLRP3 inflammasome-mediated inflammation might be a promising target for the treatment of gout arthritis.

Humanin (HNG) is a peptide first discovered by Professor Hashimoto in Keio University during their investigations on brain tissues isolated from Alzheimer’s Disease patients. It has been proved to inhibit the accumulation and deposition of Aβ [13,14]. Later investigations reveal that after replacing the Ser with Gly on the 14th site, the protective property of the peptide is enhanced 1000-fold, it is then termed S14G-HNG [15,16]. Recently, S14G-HNG has been reported to exert significant anti-inflammatory effects [17]. However, whether S14G-HNG exerts a protective effect in gouty arthritis remains unknown. In this study, we aimed to investigate its benefits on MSU crystals-induced gout arthritis and explore its potential clinical application in treating gout arthritis.

Materials and methods

Animal experiments

Forty C57BL/6 mice (male, 4-weeks-old) were obtained from the laboratory animal center of Zhejiang University (Hangzhou, China) and divided into 4 groups: the vehicle (Veh) group, model (MSU) group, colchicine (Col) group, and S14G-HNG group. Colchicine (Aladdin, Shanghai, China) was used as a positive control. Mice in the Col group were given 0.3 mg/kg colchicine-water solution and in the HNG (Invivochem, Guangzhou, China) group were intra-peritoneally administered with 3 or 6 mg/kg body weight for 7 consecutive days. To establish the model, mice in all groups, except for the Veh group, were subject to an articular luminal injection of 0.1 mL phosphate-buffered saline containing 25 mg/mL MSU crystals solution to the right knee on day 5 after administration.

Gait analysis

To conduct Gait analysis, black ink was applied to the ventral surface of the mice’s rear feet. Mice were then allowed to walk the full length of a sheet of paper. Footprints made by the right injected leg were compared to the ones made by the left uninjected ones to evaluate weight-bearing during movement. The Gait score was calculated according to the standards described previously [18].

Synovitis scoring

Based on a previous assessment, Hematoxylin-eosin (HE) staining was used for grading two synovial membrane features (thickening of synovial lining cell layer and inflammation). The scores ranged from 1 to 6, with samples divided into those with none (1), low-grade (2–3), and high-grade (4–5) synovitis [19].

Myeloperoxidase (MPO) activity

Total proteins were extracted from articular luminal synovial tissues using the radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich, USA), followed by centrifugation. Then, 4-fold volume of MPO assay buffer was used to resuspend the deposition, followed by centrifugation at 13,000 × g at 4 °C for 10 min. The supernatant was collected and transferred into tubes placed on ice, followed by being assayed with an MPO activity assay kit (Abcam, Cambridge, UK) with the absorbance at 460 nm, measured with a microplate reader (Bio-Rad, California, USA). The activity of MPO was expressed as mU/mg protein.
Cell isolation, treatment, and transduction with lentiviral SIRT1

Primary BMDMs were isolated from the bone marrow of C57BL/6 mice (n = 10). Briefly, the bone marrow was collected and rinsed in PBS with a 1 ml syringe. After centrifugation at 300 × g for 5 min, the cell pellet was resuspended in 5 ml red blood cells lysis buffer for 3 min to lyse the erythrocytes. Contaminating cells were removed by extensive washing, taking advantage of the rapid adherence of macrophages. Isolated BMDMs were cultured in Dulbecco’s modified eagle medium (DMEM):F12 medium supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, and murine recombinant CSF1 (10 ng/ml; PeproTech, 351–02). BMDMs (1 × 10⁶ cells/well in a 12-well-plate) were primed with LPS (1 μg/ml) for 3 h [20].

For the knockdown of SIRT1, specific siRNA was designed in Genscript (Nanjing, China) and packed in the lentivirus particle (lentiviral SIRT1), with si-NC taken as the negative control. Lentiviral SIRT1 was transfected into BMDMs together with the transfection reagent, lipofectamine 3000 (Life Technologies, California, USA) for 48 hours, followed by identifying the knockdown efficacy using the Western blotting assay.

Mitochondrial reactive oxygen species (ROS) measurement

MitoSOX Red assay was utilized to evaluate the production of mitochondrial ROS. In brief, cells were collected and incubated solution for half an hour with 5 μM MitoSOX at 37°C and 5% CO₂. Images were captured with a fluorescent microscope (Axiovert 200 M; Carl Zeiss, Thornwood, NY) to determine the production of mitochondrial ROS.

Malondialdehyde (MDA) measurement

The concentration of MDA in cells was determined using a commercial MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer’s instructions.

Real-time-PCR

Total RNAs were isolated from BMDMs with the Trizol solution (Life, New York, USA) and further transcribed into cDNA using the Moloney Murine Leukemia Virus Reverse transcriptase (Fermentas, Vancouver, Canada), followed by performing the PCR reaction with the ABI 7500 real-time thermocycler (Applied Biosystems, California, USA) under the following amplification conditions: 95°C for 10 s, 60°C for 30 s, and 40 cycles; and 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 30 s. Then, the LightCycler1.5 (Applied Biosystems, California, USA) and the AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) were used to conduct the RT-PCR, followed by determining the expression with the 2-ΔΔCt method after normalization with the expression level of GAPDH [21]. The following primers were used in this study: Nox-4 (forward: 5’-TGCCCTGTCATTGCTGT-3’; reverse: 5’-CCGGCATAAGGTAAAGGATG -3’); SIRT1 (forward: 5’-TGATTTGGCACC GACCTCG -3’; reverse: 5’-CCACACGGCTATCATCCACCG -3’); GAPDH (forward: 5’-TGACCTCAACTACATGGTCTACA -3’; reverse: 5’-CTTCCCATTTCTCGGCT TG -3’).

Western blot analysis

Following extracting total proteins from BMDMs with the lysis buffer, the bicinchoninic acid (BCA) kit (ZIHER, Guangdong, China) was utilized to quantify the concentration of proteins, which were then loaded and separated by the 12% SDS-PAGE [22]. The proteins were then transferred onto the PVDF membrane (Invitrogen, California, USA), incubated with 5% BSA, and then with the primary antibody against NOX-4 (1:1000, Affinity, Melbourne, Australian), IRT1 (1:1000, Affinity, Melbourne, Australian), NLRP3 (1:1000, Affinity, Melbourne, Australian), β-actin (1:1000, Affinity, Melbourne, Australian). After overnight incubation, the membrane was washed 3 times and incubated with the secondary antibody (1:2000, Affinity, Melbourne, Australian), followed by being exposed to the ECL solution. Lastly, the Image J software was utilized to visualize the bands.

Enzyme-linked immunosorbent assay (ELISA)

The release of IL-18 and IL-1β was determined using the ELISA assay (Elabscience, Wuhan, China). In brief, the supernatant and standards were collected and seeded in the 96-well plate, then incubated for 1 h at 37°C. After removing the medium, the conjugate
solution was added to be incubated for 30 min, followed by adding the TMB solution to be incubated for 15 min [23]. The stop solution was then added to terminate the reaction, and the absorbance at 450 nm was measured with the microplate reader (Bio-Rad, California, USA) to determine the protein concentration.

**Statistical analysis**

Each experiment was repeated three times. Data achieved in the present study were presented as mean ± S.D. and analyzed using the GraphPad software. The Student’s t-test was used to analyze the difference between the 2 groups and the one-way ANOVA method was used to analyze the differences among groups. P < 0.05 was considered a significant difference.

**Results**

In this study, we clarified the biological role of S14G-HNG in MSU crystals-induced gouty arthritis. We first conducted the behavioral assay, assessed the pathological status, and measured MPO activity in the articular luminal synovial tissues. Secondly, we investigated the beneficial effects of S14G-HNG on MSU crystals-induced oxidative stress and NLRP3 inflammasome activation in BMDMs. Lastly, we proved that the protective effects of S14G-HNG are mediated by SIRT1.

**S14G-HNG improved Gait score in gouty arthritis mice**

After modeling and treatments, we first conducted the behavioral assay in gouty arthritis mice. The Gait score was significantly elevated 12 (Figure 1 (a)) and 24 hours (Figure 1(b)) post MSU crystals injection. However, in both the Col and S14G-HNG groups, it was reduced, indicating the alleviation, in part, of the state of arthritis by the administration of S14G-HNG.

**S14G-HNG ameliorated the pathological status of articular luminal synovial tissues on day 7 in gouty arthritis mice**

The elevated synovitis score in the MSU group was dramatically decreased in the Col and S14G-HNG groups (Figure 2). These data reveal that the pathological changes in gouty arthritis mice were significantly ameliorated by S14G-HNG.

**S14G-HNG reduced MPO activity in articular luminal synovial tissues in gouty arthritis mice**

MPO activity is an important neutrophil infiltration biomarker. We analyzed the MPO activity in articular luminal synovial tissues isolated from each animal. We found that the MPO activity (Figure 3) was significantly increased from 936.5 mU/mg protein to 2356.7 mU/mg protein in the MSU group. Thereafter, a dramatic decline to 1036.7 mU/mg protein and 1355.9 mU/mg protein in the Col and S14G-HNG groups, respectively.

![Figure 1. S14G-humanin (HNG) improved Gait score in gouty arthritis mice. (a). Gait score after 12 hours of MSU crystals injection; (b). Gait score after 24 hours of MSU crystals injection (***, P < 0.0001 vs. vehicle group; ##, ###, P < 0.005, 0.001 vs. MSU group).](image-url)
was observed. This indicates that the neutrophil infiltration induced by the MSU crystals was dramatically reversed by S14G-HNG.

**S14G-HNG attenuated MSU crystals-induced oxidative stress in BMDMs**

Oxidative stress has been proven to be involved in the pathological changes induced by MSU crystals in the development of gout. We performed the *in vitro* assay to further verify the therapeutic mechanism of S14G-HNG. After LPS priming, the BMDMs were treated with S14G-HNG (25, 50 μM) for 2 hours. Thereafter, the cells were stimulated with MSU crystals (500 μg/mL) for 24 hours. We then assessed the state of oxidative stress by measuring the mitochondrial ROS and MDA levels in the BMDMs. The mitochondrial ROS levels (Figure 4(a)) were significantly elevated by the MSU crystals but dramatically decreased by 25 and 50 μM S14G-HNG. In addition, the increased level of MDA in the MSU group was pronouncedly suppressed by 25 and 50 μM S14G-HNG (Figure 4(b)). These data collectively show that the oxidative stress in MSU crystals-treated BMDMs was greatly ameliorated by S14G-HNG.

**S14G-HNG reduced the MSU crystals-increased expression of NOX-4 in BMDMs**

NOX-4 is an important inflammation and oxidative stress mediator. We found that the upregulated NOX-4 (Figure 5(a,B)) expression
observed in MSU crystals-treated BMDMs was dramatically downregulated by 25 and 50 μM S14G-HNG, indicating an inhibitory regulatory effect of S14G-HNG against excessive NOX-4 expression.

**S14G-HNG inhibited MSU crystals-induced activation of the NLRP3 inflammasome in BMDMs**

The NLRP3 inflammasome mediates the production of IL-18 and IL-1β to initiate inflammatory reactions. As shown in Figure 6(a), the dramatically increased
expression level of NLRP3 in MSU crystals-treated BMDMs was greatly repressed by 25 and 50 μM S14G-HNG. The MSU crystals significantly elevated the secretion of IL-1β (Figure 6(b)) from 112.5 pg/mL to 451.7 pg/mL. This was then declined to 322.7 and 251.7 pg/mL by 25 and 50 μM S14G-HNG, respectively. In addition, the secretions of IL-1β (Figure 6(c)) in the control, MSU crystals, 25, and 50 μM S14G-HNG groups were 163.3, 626.8, 476.6, and 336.5 pg/mL, respectively. These data collectively reveal that the NLRP3-mediated inflammation in MSU crystals-treated BMDMs was drastically suppressed by S14G-HNG.

**S14G-HNG restored MSU crystals-induced reduction of SIRT1 in BMDMs**

SIRT1 is a transcriptional factor involved in the regulation of the NLRP3 pathway [24], which was further investigated in the present study. We found that SIRT1 was significantly downregulated in MSU crystals-treated BMDMs at both mRNA (Figure 7(a)) and protein (Figure 7(b)) levels. The association between the regulatory effect of S14G-HNG and SIRT1 was then indicated by the upregulation of SIRT1 by 25 and 50 μM S14G-HNG.

**Silencing of SIRT1 abolished the protective effects of S14G-HNG and restored the effects of MSU crystals on NLRP3 inflammasome activation**

To further verify that SIRT1 was involved in the regulatory mechanism of S14G-HNG, cells were transduced with lentiviral- SIRT1 shRNA, followed by stimulation with S14G-HNG (50 μM) for 2 hours, and MSU crystals (500 μg/mL) for 24 hours. The efficacy of SIRT1 knockdown was confirmed by the Western blotting assay (Figure 8(a)). The elevated mRNA level of NLRP3 in MSU crystals-treated BMDMs was significantly suppressed by S14G-HNG, which was greatly reversed by the knockdown of SIRT1 (Figure 8(b)). The production of IL-1β (Figure 8(c)) in the control, MSU, S14G-HNG, and S14G-HNG+siR-SIRT1 groups was determined as 121.6, 432.6, 241.3, and 453.8 pg/mL, respectively. In addition, the secretion of IL-1β (Figure 8(d)) was dramatically elevated from 155.2 to 682.3 pg/mL in MSU crystals-treated BMDMs, greatly repressed to 395.6 pg/mL by S14G-HNG, then after the knockdown of SIRT1, it was reversed to 621.7 pg/mL.

**Discussion**

The activation and infiltration of neutrophils is an important pathological step of gouty arthritis. In the synovial fluid of healthy subjects, neutrophils are rarely observed. In gouty arthritis patients, however, the synovial fluid and membranes of their joints exhibit excessive accumulation and infiltration of neutrophils. The infiltrated neutrophils facilitate the progression of inflammation by swallowing MSU crystals and secreting inflammatory factors, such as lysosomal enzymes, oxygen free radicals, arachidonic acids, IL-1β, and IL-18 [25,26]. Consistent with the observations in the present study, the increased infiltration of inflammatory cells and enhanced MPO activity have been observed in MSU crystals-stimulated animals. After treatment with S14G-HNG and the positive control (colchicine), the infiltration of inflammatory cells and increased MPO activity were significantly repressed. These pathological alleviations, collectively with the declined Gait score, reveal
the protective effects of S14G-HNG against MSU crystals-induced symptoms of gouty arthritis.

The progression of normal physiological metabolism is often accompanied by the release of a small amount of ROS which interact with the anti-oxidative reagents to maintain the redox equilibrium. When this balance is broken by external stimuli, the excessive production of ROS is facilitated to attack the bioactive macromolecules and disrupt the normal cellular structure, impacting cellular metabolism and function [27]. MDA is the terminal product of lipid peroxidation which...
reflects the degree of disruption induced by ROS [28]. Recently, the activation of oxidative stress has been reported to be involved in the pathological changes in gout induced by MSU crystals [29]. In the present study, we found that the activation of oxidative stress in BMDMs was significantly induced by stimulation with MSU crystals, accompanied by the upregulation of NOX-4, an important mediator of oxidative stress [30]. After treatment with S14G-HNG, the state of oxidative stress was significantly alleviated, verifying the protective effect of S14G-HNG against MSU crystals-induced injury on BMDMs.

Under the stimulation of internal and external factors, the NLRP3 inflammasome is packaged and activated, which induces the transformation from inactivated pro-caspase-1 to activated caspase-1. Subsequently, IL-18 and IL-1β mature from pro-IL-18 and pro-IL-1β, which then aggravate the inflammatory cascade reaction [31,32]. It is reported that the activation of the NLRP3 inflammasome is closely associated with the pathogenesis of multiple inflammatory diseases, such as type II diabetes [33], atherosclerosis [34], Alzheimer’s Disease [35], and gout [36]. We found that the activation of the NLRP3 inflammasome was observed in MSU crystals-treated BMDMs, accompanied by the excessive production of IL-18 and IL-1β. After treatment with S14G-HNG, the activity of the NLRP3 inflammasome and the inflammation were significantly repressed, indicating that the therapeutic function of S14G-HNG might be related to the inhibition of the NLRP3 inflammasome. SIRT1 is a type III histone deacetylase induced by NAD. It is reported to be involved in multiple types of biological progressions, such as metabolism, oxidative stress, and inflammation [37]. Recently, SIRT1 has been reported to exert anti-inflammatory effects by suppressing the activity of the NLRP3 inflammasome [38,39]. We found that the downregulated SIRT1 in MSU crystals-

**Figure 8.** Silencing of SIRT1 abolished the protective effects of S14G-humanin (HNG) restored MSU crystals in NLRP3 inflammasome activation. Cells were transduced with lentiviral- SIRT1 shRNA, followed by stimulation with HNG (50 µM) for 2 h and MSU crystals (500 µg/mL) for 24 hours. (a), Western blot revealed successful knockdown of SIRT1; (b), The levels of NLRP3; (c), The levels of IL-18; (d). The levels of IL-1β (****, P < 0.0001 vs. vehicle group; ##, P < 0.005 vs. MSU group; &&, P < 0.001 vs. MSU+HNG group).
treated BMDMs was significantly reversed by S14G-HNG. In addition, the protective effects of S14G-HNG against MSU crystals-induced NLRP3 inflammasome activation were dramatically abolished by the knockdown of SIRT1, indicating that SIRT1 is an important mediator involved in the regulatory effect of S14G-HNG. In future work, the specific target of S14G-HNG in regulating the expression level of SIRT1 will be investigated to better understand the interaction between S14G-HNG and SIRT1.

Conclusion

According to the experimental results, we demonstrate that intervening with S14G-HNG can effectively ameliorate the pathological status of articular luminal synovial tissues and reduce MPO activity. Furthermore, S14G-HNG treatment significantly attenuated oxidative stress, expression of NOX-4, and activation of the NLRP3 inflammasome in MSU crystals-challenged BMDMs. Mechanistically, S14G-HNG treatment rescued MSU crystals-induced reduction of SIRT1 in BMDMs. Taken together, our data reveal that S14G-HNG protected against MSU crystals-induced gouty arthritis by regulating SIRT1, suggesting that S14G-HNG might be a promising therapeutic agent for the treatment of gouty arthritis.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Consent to publication

All the authors have read and approved the final submission of this study.

Ethical statements

The protocol of this study was approved by the ethical committee of The Second Affiliated Hospital of Harbin Medical University.

Data availability statement

Data of this study are available upon reasonable request to the corresponding authors.

Author contribution

Jihui Zhang contributed to the study design. Jihui Zhang, Hongwei Lei, and Xiul Li performed the experiments, summarized all data, and performed the statistical analysis. Jihui Zhang drafted the manuscript and supervised the entire study. All authors critically reviewed the manuscript and approved the final draft.

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ORCID

Jihui Zhang http://orcid.org/0000-0002-6236-2547

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