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Resveratrol attenuates the MSU crystal-induced inflammatory response through the inhibition of TAK1 activity

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ABSTRACT

TAK1 is closely associated with the NF-κB and MAPK signaling pathways. In the present study, we aimed to explore the relationship between TAK1 and gout as well as the effects of resveratrol on TAK1 activity and MSU crystal-induced inflammation. The expression levels of total TAK1 and phosphorylated TAK1 in gout patients were detected by western blotting. The influence of resveratrol on TAK1 activity and MSU crystal-induced inflammation was investigated in THP-1 cell and murine models of gout. The results showed that TAK1 and p-TAK1 were highly expressed in gouty arthritis patients. MSU crystals accelerated the expression of TAK1 and p-TAK1 in human PBMCs. The anti-inflammatory effects of resveratrol on MSU crystal-induced inflammation in vitro and in vivo included the alleviation of pro-inflammatory cytokines, inflammatory cell recruitment and foot swelling. Resveratrol limited the activation of TAK1 and its downstream signaling pathway, including the degradation of IκBα, the activation of NF-κB P65 and the phosphorylation of P38 and JNK. In conclusion, resveratrol may have a potential therapeutic effect on gouty arthritis by inhibiting TAK1 activity.

1. Introduction

Gout is one of the most severe and common forms of inflammatory arthritis, being characterized by the deposition of monosodium urate (MSU) crystals in articular joints and periarticular tissues [1]. Epidemiological evidence implies that the morbidity of gout is dramatically increasing in developed and developing countries [2]. A thorough understanding of how MSU crystals evoke inflammation is essential to reveal novel pathways and pharmacological targets. Further curbing unconventionally painful and disabling gout flares is desirable [3].

Transforming growth factor-β activated kinase 1 (TAK1, also called MAP3K7) is a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family [4]. TAK1 activation is closely associated with its phosphorylation primarily on Thr187 and Thr184 [5,6]. Several other known TAK1 cues, including IL-1β, TNF-α and TLR4, have been proven to be activated and to play an important role in gout [7]. Activated TAK1, in turn, activates the NF-κB and MAPK signaling pathways, which are essential for MSU crystal-induced inflammation. In particular, NF-κB is critical for the priming of the NLRP3 inflammasome in MSU crystal-induced inflammation [8]. TAK1 has been implicated in a number of pathophysiologic processes, including central nervous system (CNS) autoimmune inflammation, arthritis, and colitis [9–11].

Resveratrol (*trans*-3, 5, 4′-trihydroioxystilbene, Res) is a polyphenolic phytoalexin with excellent antioxidant and anti-inflammatory properties. A previous study showed that resveratrol can significantly inhibit TAK1 activation by targeting at both the N161 and A107 residues [12]. However, resveratrol is hydrophobic and sensitive to external agents such as air, light and oxidative enzymes, strongly limiting its stability and bioavailability in vivo. To overcome these limitations, many studies have examined the formation of inclusion complexes of resveratrol and 2-hydroxypropyl-β-cyclodextrin to increase the solubility of resveratrol in water and further protect it against hydrolysis, oxidation, and photodecomposition [13–15]. In the current study, we dissolved resveratrol in a 2-hydroxypropyl-β-cyclodextrin solution to study whether it can inhibit MSU crystal-induced inflammation.

We detected the expression levels of TAK1 and p-TAK1 in patients with acute and non-acute gouty arthritis. The effects of resveratrol were also evaluated in cell and murine gout models. Our results suggest that TAK1 activity might be closely associated with the inflammatory response in gout and that resveratrol can inhibit MSU crystal-induced

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inflammation by down-regulating TAK1 activity. To our knowledge, this study is the first to indicate that TAK1 may be involved in MSU crystal-induced inflammation and that resveratrol may have a potential therapeutic effect on gouty arthritis.

2. Materials and methods

2.1. Patients and healthy controls

A total of 44 male patients with primary gouty arthritis (GA) were involved in this study. Classification was carried out according to the gout criteria proposed by the American College of Rheumatology (ACR). The gouty patients were divided into acute (n = 22) and non-acute groups (n = 22), and a healthy control group of individuals without hyperuricemia, metabolic syndrome or other chronic diseases was included (n = 22). PBMCs were separated from peripheral blood samples by centrifugation using Ficoll-Paque Plus solution (Amersham Biosciences, Uppsala, Sweden), after which they were used for cell culture or were stored at −80 °C for protein extraction. This study was approved by the Affiliated Hospital of North Sichuan Medical College and informed consent was obtained from all participants.

2.2. Preparation of MSU crystals

MSU crystals were prepared as described by Scanu et al. [16]. Briefly, 1 g of uric acid was dissolved in 194 ml of deionized water, and 6 ml of 1 N NaOH was added, followed by heating to 60 °C. The pH value of the fluid solution was adjusted to 8.9 with HCl, and it was allowed to crystallize overnight at room temperature. The precipitate was filtered from the solution and dried at 40 °C. The crystal sizes were validated using polarizing light microscopy. Endo-toxin levels in the MSU crystal suspensions were checked by Limulus amoebocyte cell lysate assay (Sigma-Aldrich).

2.3. Isolation of PBMCs, cell culture, and MSU crystal stimulation

Freshly collected PBMCs from healthy controls were plated at a density of 5×10^5 cells/ml on 35-cm dishes with complete RPMI 1640 culture medium containing 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, supplemented with 10% fetal bovine serum. MSU crystals suspended in PBS were added to the culture plates, and the cells were cultured for the indicated times at 37 °C in a 5% CO_2 humidified incubator.

2.4. Resveratrol treatment of THP-1-derived macrophages simultaneously stimulated with MSU crystals

Resveratrol (purity ≥ 99%) was purchased from Sigma (St. Louis, MO, USA). (2-hydroxypropyl-β-cyclodextrin, purity > 98%) was purchased from Innochem (Beijing, China). THP-1 monocytes were suspended in complete culture medium and seeded in 24-well culture plates (2×10^5 cells/ml/well). The cells were then treated with 100 ng/ml phorbol myristate acetate (PMA) for 24 h to obtain THP-1-derived macrophages. Resveratrol at concentrations of 5, 10, and 20 μM showed no cytotoxicity against THP-1-derived macrophages. THP-1-derived macrophages were added to a 200 μg/ml MSU crystal suspension and were simultaneously treated with different concentrations of resveratrol for 24 h (the control contained a 2-hydroxypropyl-β-cyclodextrin solution). The ratio of the number of molecules of resveratrol to 2-hydroxypropyl-β-cyclodextrin was 1:3, and the supernatants were harvested and stored at −80 °C for cytokine detection by ELISA. The collected cells were prepared for protein extraction and western blotting.

2.5. Resveratrol administration MSU crystal-induced murine peritonitis

Peritonitis was induced by the intraperitoneal injection of an MSU crystal suspension (3 mg in 120 μl of PBS) into C57BL/6 male mice (6 to 8 weeks old). After 1 h, the mice were treated with a medium dose of resveratrol (15 mg/kg, intraperitoneal injection of 100 μl of 3 μg/μl resveratrol solution) [12]. The control mice were treated with a 2-hydroxypropyl-β-cyclodextrin solution. After 6 h, the number of peritoneal cavity exudate cells was counted by hemocytometer, and the peritoneal lavage fluids were collected for the detection of cytokine levels by ELISA.

2.6. Resveratrol intervention in MSU crystal-induced murine gouty arthritis

C57BL/6 male mice (six to eight weeks old) were anesthetized by intraperitoneal injection (50 mg/kg sodium pentobarbital) and were then injected intra-articularly with an MSU crystal suspension into the right foot pad (1 mg in 40 μl of PBS) [7]. Meanwhile, the control mice were injected with an equal amount of sterile PBS into the left foot pad. The mice were treated with resveratrol at a dose of 15 mg/kg one hour after MSU crystal administration. The control mice were treated with a 2-hydroxypropyl-β-cyclodextrin solution. At 6 h of MSU crystal treatment, joint swelling was measured, and the mice were euthanized. The articular tissues were removed and preserved for protein extraction or fixed with formalin for histopathologic analysis.

2.7. Foot thickness evaluation

Joint swelling was examined using a Digimatic caliper (Mitutoyo, Kawasaki, Japan), the minimum accuracy of which is 0.01 mm [17]. The degree of joint swelling was defined as the ratio of the foot pad thickness of the inflammatory tissue over that of normal joint tissue; values exceeding 1.10 were considered to be inflammation.

2.8. Histological studies

Murine joint tissue sections were stained with H&E. Images were obtained using a light microscope (Olympus, Tokyo, Japan) with 100× and 200× objectives and they were analyzed with image processing software (Olympus).

2.9. Elisa

The levels of cytokines in sera, cell culture supernatants and lavage fluids were detected by ELISA (Neobioscience kit, Shenzhen, China) according to the manufacturer's instructions.

2.10. Western blotting analysis

The proteins were transferred onto PVDF membranes through wet transfer. After blocking, the membranes were incubated with the specified primary antibodies at 4 °C overnight, including anti-TAK1 (catalog number RT1377), anti-p-TAK1 (Thr 187 and Thr184, catalog number 4531, from Cell Signaling Technology), anti-IκBα (catalog number ET1603-6), anti-p-P65 (catalog number 3033, from Cell Signaling Technology), anti-p-ERK1/2 (catalog number RT1206), anti-p-P38 (catalog number RT1483) and anti-p-JNK (catalog number ET1609-42). The membranes were then incubated with secondary antibodies (1:5000) for 1 h at room temperature and exposed using a gel imaging system and chemiluminescence kit. Band-intensity was quantified using ImageJ software.

2.11. Statistical analysis

The data were analyzed using GraphPad PrismV.5.00 software and are expressed as the mean (± SEM) or median (range) of three

Table 1
Clinical characteristics of the subjects.

	AGA group (n = 22)	NAGA group (n = 22)	HC group (n = 22)
Age(years)	43.76 ± 11.25	41.3 ± 9.87	47.25 ± 12.13
BMI(kg/m^2)	26.38 ± 3.21[a]	27.7 ± 2.85[a]	25.32 ± 3.43
UA(μmol/L)	591.6 ± 115.38[a,b]	487.36 ± 103.45[a]	347.02 ± 46.74

AGA (acute gouty arthritis), NAGA (non-acute gouty arthritis), HC (health control), BMI (body mass index), UA (uric acid).
[a] $P < 0.01$ (in comparison with the HC group).
[b] $P < 0.05$ (in comparison with the NAGA group).

independent experiments (GraphPad Software, San Diego CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical and laboratory data of the subjects

In this study, gout cases were age-matched with control individuals ($P < 0.05$) (Table 1). Large difference in the levels of UA and BMI were observed among the acute gouty arthritis (AGA), non-acute gouty arthritis (NAGA) and healthy control (HC) groups ($P < 0.05$). The UA concentration was considerably higher in the AGA patients compared to that in the NAGA patients or HC subjects ($P < 0.01$), and higher levels of UA were also observed in the NAGA group compared to that in the HC group ($P < 0.05$). The AGA and NAGA patients had much higher BMIs than those in the HC group ($P < 0.05$).

3.2. TAK1 is highly expressed and activated in GA patients

To determine whether the expression level of total TAK1 is increased and activated in gouty arthritis, we examined the protein levels of TAK1 and phosphorylated TAK1 (p-TAK1, Thr-187 and Thr-184) via western blotting in PBMCs. Almost no difference in the total TAK1 protein level was found between patients with acute and non-acute gout, but the total TAK1 and p-TAK1 protein levels in the GA patients were higher than those in the healthy controls. The level of p-TAK1 in the AGA patients was markedly higher than that in the NAGA patients (Fig. 1).

3.3. MSU crystals can induce the expression of TAK1 and p-TAK1 in PBMCs

Because TAK1 and p-TAK1 were highly expressed in the PBMCs of AGA and NAGA patients, we examined whether endogenous TAK1 could be directly triggered by MSU crystals in PBMCs. Freshly isolated PBMCs were stimulated with different concentrations (100, 200 and 400 μg/ml) of an MSU crystal suspension for 24 h. Both the TAK1 and p-TAK1 protein levels increased in a dose-dependent manner in THP-1 cells upon treatment with MSU crystals (Fig. 2).

3.4. Resveratrol relieves MSU crystal-induced inflammation in THP-1-derived macrophages

A previous study reported that resveratrol could suppress the expression of TAK1 to further prevent inflammation. To explore whether resveratrol affects the expression of TAK1 and alleviates MSU crystal-induced inflammation, THP-1 cells were simultaneously treated with a 200 μg/ml MSU crystal suspension and different doses of resveratrol for 24 h. Resveratrol downregulated the protein level of p-TAK1 but not that of TAK1 (Fig. 3a–c). Resveratrol treatment also significantly inhibited the secretion of IL-1β and TNF-α (Fig. 3d, e). These data imply that resveratrol can efficiently prevent TAK1 activation and attenuate the MSU crystal-induced inflammatory response.

3.5. Resveratrol limits MSU crystal-induced murine peritonitis and gouty arthritis

To test the effects of resveratrol on MSU crystal-induced murine peritonitis, total cell numbers were measured from lavage fluids in a peritonitis mouse model. Resveratrol treatment greatly reduced the number of inflammatory cells in lavage fluid compared to that of the vehicle injected mice (Fig. 4a). We also found that the production of IL-1β and TNF-α was decreased in the peritoneal cavity lavage fluid from the mice in the resveratrol treatment group (Fig. 4b, c).

To evaluate the effects of resveratrol on MSU crystal-induced murine gouty arthritis, foot thickness was measured and joint tissue was histopathologically examined. Foot swelling was ameliorated in the gouty arthritis mice after treatment with resveratrol (Fig. 4d–f). This result was in line with the histopathological data from joint tissue sections. HE staining showed that resveratrol administration markedly relieved inflammatory cell infiltration in the joint tissue sections (Fig. 4i, j), in the vehicle treated group, many inflammatory cells were present (Fig. 4g, h).

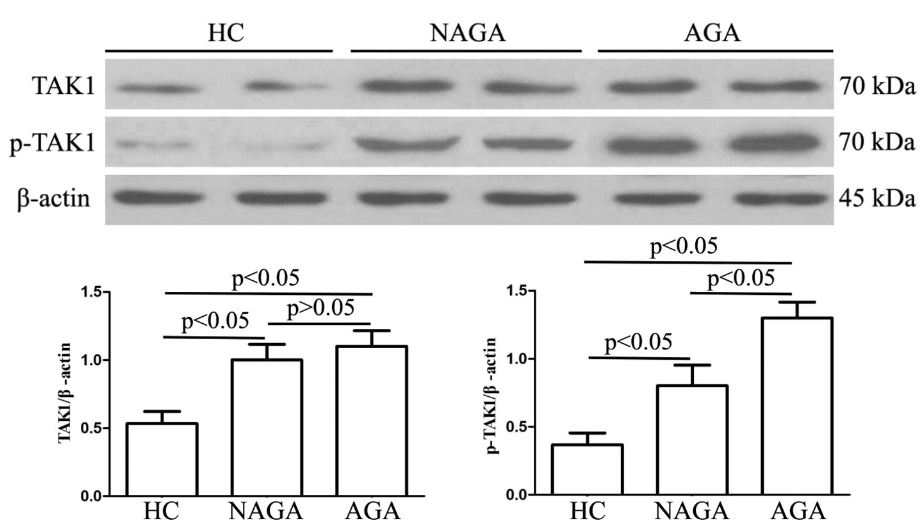

Fig. 1. TAK1 and p-TAK1 expression levels in the HCs, NAGA patients and AGA patients. The protein levels of TAK1 and p-TAK1 in the PBMCs were determined by western blotting. Band density was normalized to the expression of β-actin.

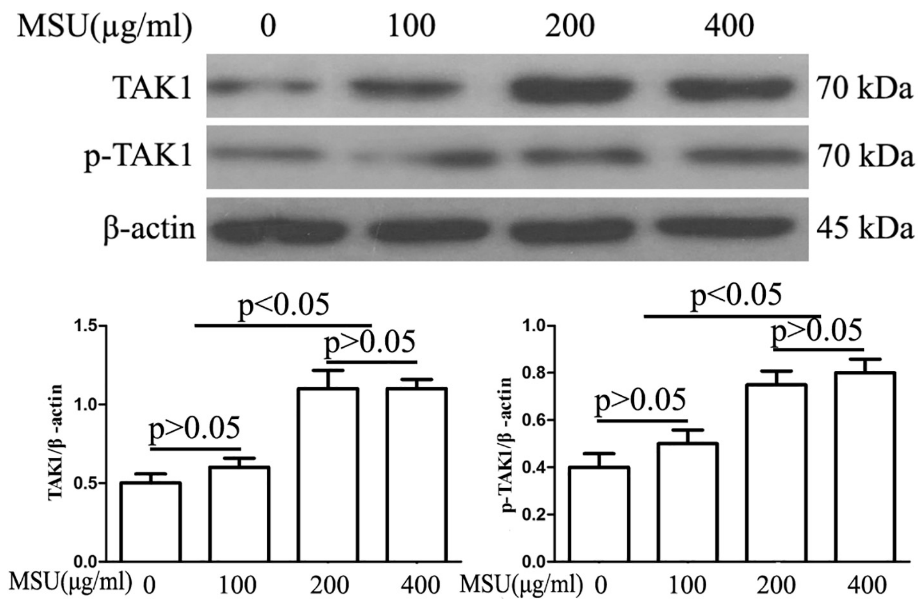

Fig. 2. MSU crystals induced the expression of TAK1 and p-TAK1 in the PBMCs. TAK1 and P-TAK1 protein levels were measured by western blotting analysis. Band density was normalized to the expression of β-actin.

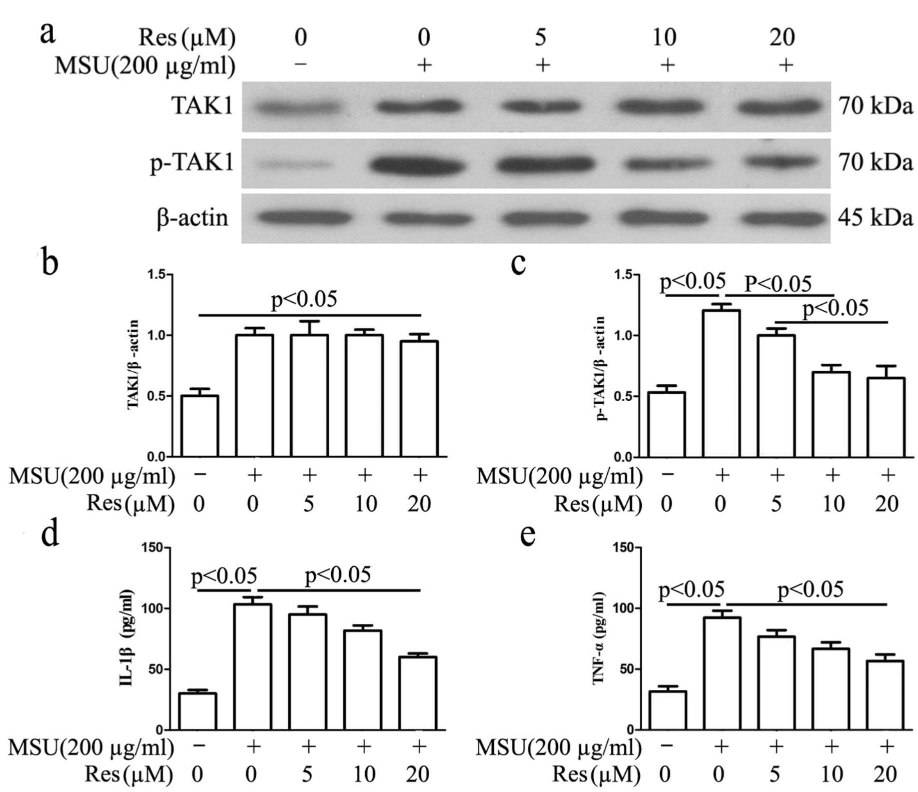

Fig. 3. Effects of resveratrol on the TAK1 and p-TAK expression levels and inflammatory cytokine production in THP-1-derived macrophages. a-c Western blots examining the TAK1 and p-TAK1 protein levels in THP-1 cells treated simultaneously with MSU crystals and Res at concentrations of 5 μM, 10 μM and 20 μM, respectively, or vehicle (2-hydroxypropyl-β-cyclodextrin solution). d ELISA to detect the levels of IL-1β and TNF-α in THP-1 cell supernatants. e ELISA to examine the level of TNF-α in THP-1 cell supernatants.

3.6. Resveratrol inhibits TAK1 activity and its downstream NF-κB and MAPK signaling pathways

To investigate whether resveratrol can inhibit TAK1 activity in gouty arthritis mice, the protein levels of TAK1 and p-TAK1 were analyzed using western blotting. Resveratrol treatment greatly lowered the protein level of p-TAK1 in the arthritis mouse model, but it did not affect the TAK1 protein level, as observed in the resveratrol-treated and vehicle-treated mice (Fig. 5). TAK1 regulates the activity of the NF-κB and MAPK signaling pathways. First, we explored the activation of the NF-κB signaling pathway, including IκBα degradation and NF-κB P65 activation. The basal level of IκBα was high in the control mice, and resveratrol inhibited MSU crystal-induced IκBα degradation (Fig. 5). Furthermore, the level of p-P65 almost returned to that of the control mice after resveratrol treatment in the arthritis mouse model (Fig. 5). We further evaluated the effects of resveratrol on the activation of members of the MAPK family by western blotting. The protein levels of p-JNK, p-ERK1/2 and p-P38 were considerably increased in the MSU crystal-induced arthritis mice (Fig. 5), resveratrol decreased the expression levels of p-JNK and p-P38, however, the level of p-ERK1/2 remained unchanged after treatment with resveratrol in the arthritis mouse model (Fig. 5).

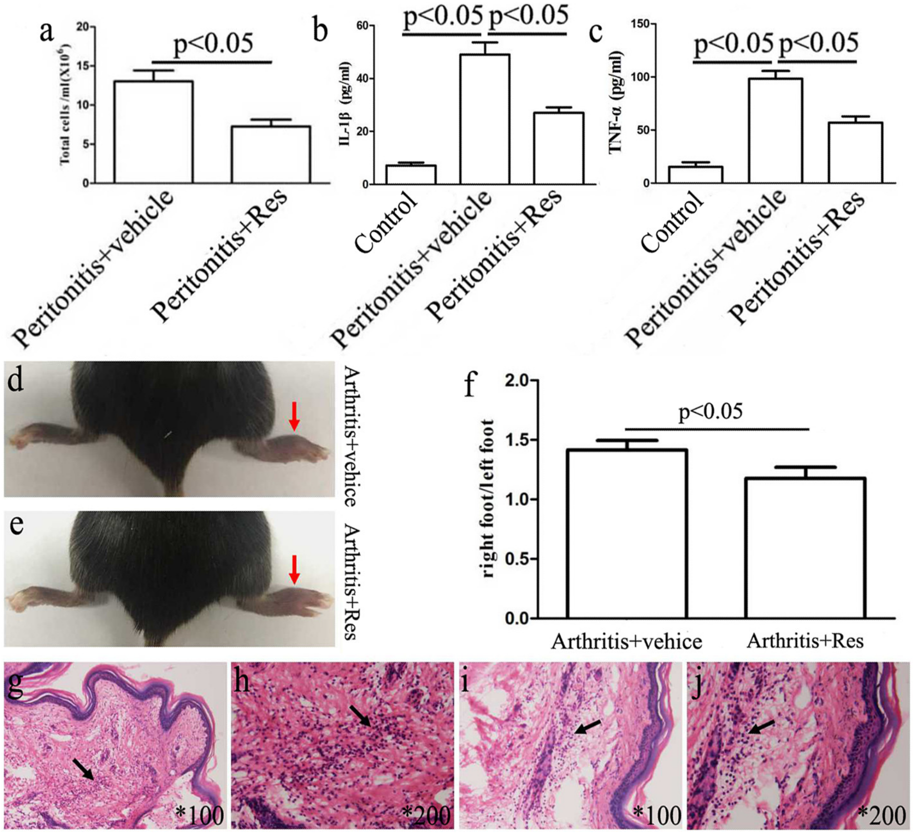

Fig. 4. Effects of resveratrol on MSU crystal-induced murine peritonitis and gouty arthritis. a Total cell numbers in the lavage fluids from the peritoneal cavity were counted by hematocytometry. b The IL-1β level in the peritoneal cavity lavage fluids was measured by ELISA. c The TNF-α level in the peritoneal cavity lavage fluids. d–f Foot thickness was evaluated in the resveratrol treatment (Arthritis + Res) and control (Arthritis + vehicle) groups. g–h Histopathological analysis of joints from the non-Res treatment group by H&E staining (100× original magnification). h 200× original magnification. Arrow indicates abundant inflammatory cells. i–j Histopathological analysis of joints from the Res treatment group by HE staining (100× original magnification). j 200× original magnification. Arrow indicates fewer inflammatory cells in the soft tissue and joint spaces. The results are representative of three independent experiments; n = 3–5 mice per group.

4. Discussion

Currently, TAK1 is considered an important therapeutic target in many types of inflammatory disease and cancer [18,19]. However, the correlation between TAK1 and gout is poorly understood. The protein levels of TAK1 and its active form p-TAK1 were aberrantly high in the PBMCs from GA patients, even in the NAGA patients (Fig. 1). In addition to the AGA patients, the level of UA in the NAGA group was higher than that in the HC group. These data suggest that serum UA may affect the expression of TAK1 and p-TAK1. Accordingly, we explored whether MSU crystals could directly stimulate the expression of TAK1 and p-TAK1 in PBMCs. PBMCs were stimulated with different concentrations of MSU crystals and the data indicated that the expression levels of TAK1 and p-TAK1 were increased in a dose-dependent manner. Based on these data, we further verified our hypothesis that the inhibition of TAK1 might control MSU crystal-induced inflammation.

A previous study reported that a natural product, resveratrol, can target TAK1 and significantly inhibit TAK1 activation to attenuate inflammation [12]. Different gouty models were used to explore the anti-inflammatory effect of resveratrol in vitro and in vivo. In MSU crystal-induced THP-1 cells and arthritis mice, resveratrol could inhibit the active form of TAK1 (p-TAK1, T187). In acute gouty arthritis, IL-1β and TNF-α act as the major pro-inflammatory cytokines and they recruit neutrophils to the synovium and joints. Resveratrol may also reduce the production of IL-1β and TNF-α in MSU crystal-induced THP-1 cells and murine peritonitis models. However, Haiyan Chen et al. reported that resveratrol has a preventive effect on MSU crystal-induced arthritis and not a therapeuticy [20]. In their study, resveratrol was dissolved in DMSO. In our study, we noticed that the mice were immediately adversely affected by treatment with resveratrol that was dissolved in DMSO, even after being injected with a low dose of resveratrol (5 mg/kg, injection of 33.3 μl of a 3 μg/μl resveratrol solution). However, when resveratrol was dissolved in a 2-hydroxypropyl-β-cyclodextrin solution, the mice appeared to be in good condition after the injection of a medium dose of resveratrol (15 mg/kg), and the treatment had good therapeutic effects and greatly inhibited the increase of foot thickness in the MSU crystal-induced arthritis mice. However, gout is not only associated with high levels of uric acid but also with genetic and environmental factors. Some gout patients have high levels of uric acid for quite a long time and do not develop gout. The gout mouse model we used was caused only by MSU crystals, so further clinical trials are needed concerning the use of resveratrol in patients with gout.

Activated TAK1 can accelerate the NF-κB and MAPK signaling pathways. NF-κB is a vital transcription factor that is involved in a variety of inflammatory response. In a resting state, NF-κB and its inhibitor (IκBα) combine to form a dimer that is located in the cytoplasm. After activation, IκBα is degraded, and NF-κB shifts from the cytoplasm to the nucleus, resulting in the transcription and expression of genes related to inflammation. In the current study, our results showed that resveratrol significantly decreased MSU crystal-induced NF-κB activation. We also found that resveratrol selectively decreased the activity of the MSU crystal-induced MAPK signaling pathway, including the phosphorylation of p38 and JNK but not ERK1/2.

In conclusion, the present study suggests that TAK1 might be a novel inflammatory mediator in MSU crystal-stimulated inflammation. Resveratrol greatly reduced IκBα degradation, and NF-κB p65 activation as well as p38 and JNK phosphorylation (Fig. 5). NF-κB is essential for the priming, assembly and activation of the NLRP3 inflammasome which is also a key step for the release of inflammatory cytokines in MSU crystal-induced inflammation [3]. Whether resveratrol affects the assembly and activation of NLRP3 inflammasome by affecting the activity of TAK1 remains to be further studied.

Ethical approval

The study protocol was approved by the Institutional Ethics Committee at the North Sichuan Medical College, and informed consent was obtained from patients.

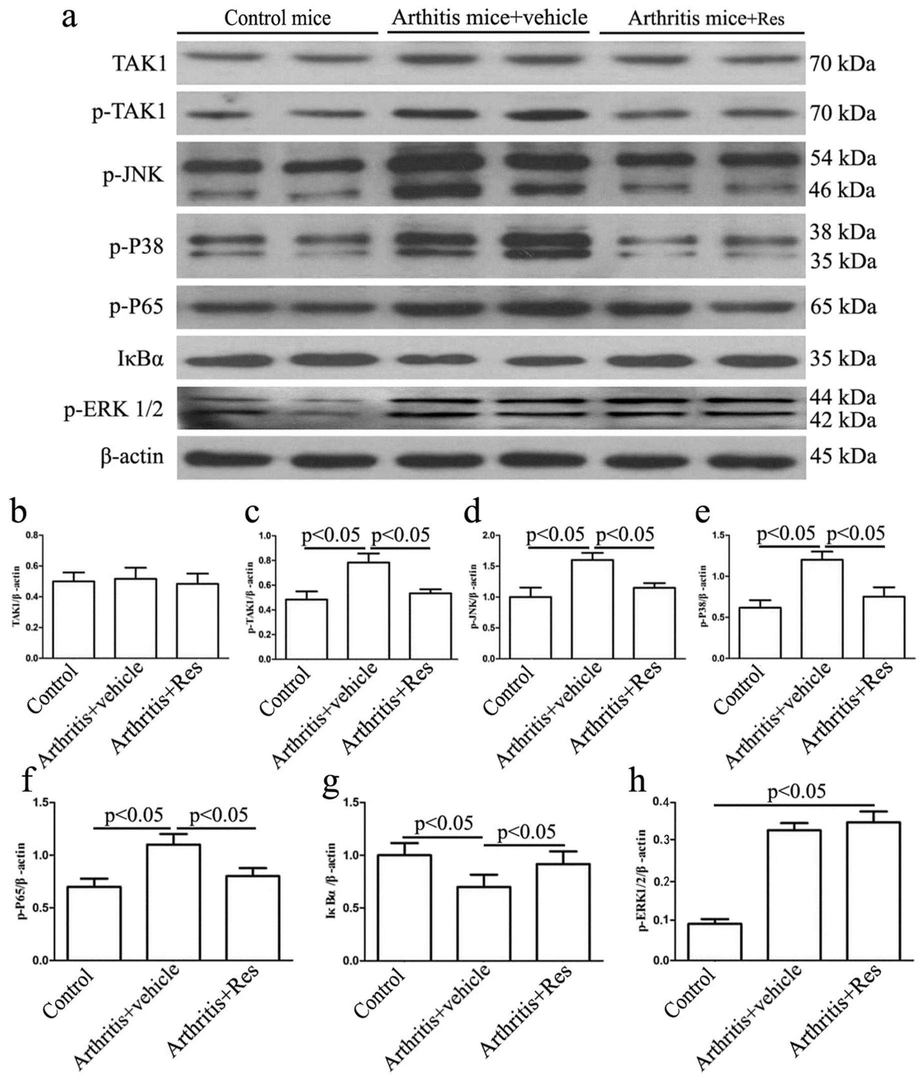

Fig. 5. Effects of resveratrol on the expression levels of TAK1, p-TAK1, p-JNK, p-P38, p-P65, IκBα and p-ERK1/2. a Protein extracted from the joint tissue of mice was reacted with anti-TAK1, anti-p-TAK1, anti-p-JNK, anti-p-P38, anti-p-P65, anti-IκBα and anti-p-ERK1/2 via western blotting. b The relative amounts of TAK1. c The relative amounts of p-TAK1. d The relative amounts of p-JNK. e The relative amounts of p- p-P38. f The relative amounts of p-P65. g The relative amounts of IκBα. h The relative amounts of p-ERK1/2. The values are presented as the mean ± SEM of three independent experiments, n = 3–5 mice per group.

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications nos. 80-23, revised 1996) and institutional ethical guidelines.

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Conflict of interest

The authors report no conflicts of interest.

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