



Inhibition of monosodium urate crystal-induced inflammation by scopoletin and underlying mechanisms

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ABSTRACT

The present study determined the anti-inflammatory activity of scopoletin in gout air pouch model and revealed the underlying mechanisms by *in vitro* assays. Monosodium urate (MSU) crystal-induced inflammation in mouse air pouch model, an experimental model for acute gout, was used to assess the efficacy of scopoletin. The neutrophil and mononuclear phagocyte numbers and MPO levels were increased significantly six hours after MSU crystal injection into the air pouch, whereas these changes were inhibited substantially upon scopoletin (100 and 200 mg/kg, i.p.) treatment. To get insight into the underlying mechanisms, the *in vitro* studies were performed to investigate the effects of scopoletin on activation of macrophages and resultant production of inflammatory mediators. The secretions of interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), prostaglandin E₂ (PGE₂) and nitric oxide (NO) were elevated in MSU crystal-stimulated RAW 264.7 cells, and scopoletin (30–300 μ M) suppressed the production of all mediators. Moreover, RT-PCR assay and western blot analysis indicated that scopoletin regulated the transcriptional level of these mediators *via* suppression of NF- κ B activation and blockade of MAPK signal pathway. Thus, the results clearly indicated that scopoletin inhibited the monosodium urate crystal-induced inflammation both *in vivo* and *in vitro*. In combination with our previous findings that scopoletin shows hypouricemic, anti-angiogenesis and pro-apoptotic activities, this compound may be a potential agent for gout therapy and could serve as a structural base for developing new drugs.

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1. Introduction

The worldwide incidence and prevalence of gout have been increasing over the last few decades, making gout far and away the most common inflammatory arthritis. Gout affects at least 1% of the population in developed countries [1]; and with the development of the economy as manifested by dietary and lifestyle changes, its prevalence appears to be climbing in China, especially in some coastal cities [2].

Gout is a progressive rheumatic disease, caused by an inflammatory response to monosodium urate (MSU) crystals deposited in joints and connective tissues as a result of an excess of uric acid in the blood or hyperuricemia. Most frequently it is characterized by recurrent attacks of acute arthritis, if left untreated or inadequately treated it may eventually progress to chronic arthropathy, tophi depositions, and nephropathy. Additionally, ~60% of the patients with gout in the USA are diagnosed with metabolic syndrome that includes diabetes, hypertension, abdominal obesity and hyperlipidemia [3]. The patients also have a higher risk of death, especially because of cardiovascular disease [4]. Thus, treatment options are limited because of the associated metabolic syndrome and

comorbid illnesses. This complex condition and the high prevalence call for more pharmacological options, both for controlling acute inflammatory arthritis as well as for long-term prophylaxis aiming to reduce serum urate levels.

Scopoletin (6-methoxy-7-hydroxycoumarin, C₁₀H₈O₄, Fig. 1) is the major active coumarin isolated from the stems of *Erycibe obtusifolia* Benth (Convolvulaceae), which is usually used for rheumatic arthritis therapy in Traditional Chinese Medicine. It has been reported to possess hypotensive [5], vasorelaxant [6], anti-oxidative [7,8], anti-thyroid and anti-hyperglycemic activities [8] which are associated with metabolic syndrome. And it has hepatoprotective effect instead of hepatotoxicity [8,9]. Importantly, scopoletin showed strong anti-inflammatory and anti-nociceptive properties by reducing 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema and acetic acid-induced visceral pain [10,11], and it could inhibit the production of inflammatory mediators in lipopolysaccharide-stimulated macrophages and human mast cell line [12–14]. By the way, scopoletin is also a weak xanthine oxidase inhibitor [15]. Taken together, scopoletin might be a candidate for the treatment of gout.

We previously reported that scopoletin exhibited significant hypouricemic activity in hyperuricemic mice induced by potassium oxonate through the inhibition of uric acid production as well as uricosuric

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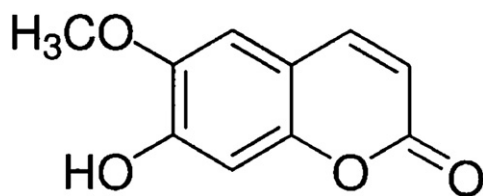


Fig. 1. Chemical structure of scopoletin (6-methoxy-7-hydroxycoumarin, $C_{10}H_8O_4$, molecular weight 192.17).

mechanisms [16]; and it showed a remarkable anti-inflammatory effect in both croton oil- and carrageenan-induced inflammatory models involving inhibition of PGE_2 and $TNF-\alpha$ overproduction, and neutrophil infiltration [17]. We also studied the anti-arthritic effect of scopoletin. Our data demonstrated that scopoletin ameliorated clinical symptoms of rat adjuvant-induced arthritis partially by preventing synovial angiogenesis and inducing apoptosis of fibroblast-like synoviocytes [18–20].

MSU crystals, the etiologic agent of gout, are potent pro-inflammatory stimuli. Within the joint, they trigger a local inflammatory reaction, leukocyte recruitment, and the production of inflammatory mediators [21], resulting in intense joint pain, swelling and erythema. Infiltration of vast numbers of neutrophils into inflamed joints is the hallmark of acute gouty arthritis. However, cellular kinetic analysis using rat air pouch model of gout indicates that an increase in monocytes/macrophages density precedes the neutrophil influx [22]. Recent studies also implicate innate cells of monocyte/macrophage lineage as playing a governing role in the pathogenesis of gout [23,24]. Mononuclear phagocytes respond to MSU crystals by activating expression of a number of pro-inflammatory genes, including those encoding $IL-1\beta$, $TNF-\alpha$, $IL-6$, $COX-2$ and $iNOS$ [25–29]. Then the local generations of inflammatory mediators act on endothelial cells and synovial cells and orchestrate the course of inflammation. Subsequent neutrophil influx into the joint space appears to execute and amplify gouty arthritis [30,31]. This study was thus designed to evaluate the *in vivo* anti-inflammatory activity of scopoletin in acute gout model and to explore the possible mechanisms involved from the view of pro-inflammatory mediator production and molecular signal pathway regulation.

2. Materials and methods

2.1. Chemicals and reagents

Scopoletin (purity >98%, by HPLC) was isolated from the stems of *E. obtusifolia* Benth which were collected at Guangxi province of China. The extraction and purification procedures were the same as described before [18]. It was suspended in 0.8% sodium carboxymethylcellulose solution (CMC-Na) in sterile endotoxin-free phosphate-buffered saline (PBS) for intraperitoneal (i.p.) injection and dissolved in DMSO then diluted with culture medium to 0.1% DMSO for *in vitro* assay. MSU was from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). It was heated at 180 °C for 2 h before suspended in sterile, endotoxin-free medium. The suspensions were ultrasonicated for 3 min before use. All solutions were verified to be free of detectable endotoxin by the *Limulus* amoebocyte lysate test (Pyrochrome; Associates of Cape Cod, Falmouth, MA). Prednisolone, bovine serum albumin (BSA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Tween-20, sodium dodecyl sulfate (SDS), dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF) were all from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA); Dulbecco's modified Eagle medium (DMEM), penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA); and newborn calf serum was from PAA Laboratories GmbH (Pasching, Austria). The other chemicals and reagents used were of analytical grade. All the glassware that was used in preparing the chemicals or reagents was sterilized by autoclaving and dried at 180 °C for 2 h.

2.2. Animals

The study was conducted using male ICR mice (25–30 g), housed in a temperature and humidity controlled room (22 ± 2 °C, $50 \pm 20\%$) under a 12-h light–dark cycle (lights on at 07:00 AM). The animals were acclimatized for one week before the experiment began with free access to food and water. The animal care and use was complied with the Provisions and General Recommendation of the Chinese Experimental Animals Administration Legislation. The experiments were performed under a license granted by the Science and Technology Department of Jiangsu Province (License Number SYXK 2007-0025), with approval from the Animal Ethics Committee of China Pharmaceutical University.

2.3. Murine air pouch model and the drug treatment

The murine air pouch is a bursal space that resembles the human synovium [32]. And the injection of MSU crystals into the pouch cavity elicits an acute inflammatory response similar to gout. Air pouches were created on the backs of the animals. Five milliliters of filtered air was injected subcutaneously to create a pseudosynovial cavity on day 0. A second air injection (3 ml) was given on day 3 to keep the pouch inflated.

The animals were divided into six groups, each comprising of eight animals. On day 6, the animals in groups 1, 2 and 3 received an i.p. injection of scopoletin (50, 100 and 200 mg/kg) suspended in 0.8% CMC-Na solution. In group 4, they received an i.p. injection of prednisolone (10 mg/kg) in 0.8% CMC-Na solution. In groups 5 and 6, they received the vehicle. After 30 min, the animals in group 6 received 1 ml PBS in the pouch, and other groups received 3 mg MSU suspended in 1 ml PBS. Six hours later, the animals were euthanized by CO_2 exposure.

2.4. Leukocyte counting and myeloperoxidase (MPO) level in the pouch exudate

The pouch exudate was collected by injecting 2 ml PBS and infiltrating leukocytes were counted with a hemocytometer. Differential leukocyte counts were measured by Wright–Giemsa staining of cytospin slides. MPO levels in the supernatants of pouch exudates were determined colorimetrically using the MPO Detection kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu Province, China).

2.5. Cell culture

Monocyte/macrophage cell line RAW 264.7, obtained from American Type Culture Collection (ATCC), were cultured in DMEM containing 10% newborn calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin and maintained at 37 °C in a 5% CO_2 humidified atmosphere.

2.6. Cell viability assay

The cytotoxic effect of scopoletin was evaluated by conventional MTT assay. RAW 264.7 cells (1×10^5 cells/well) were seeded and incubated for 2 h in 96-well microplates. Then they were exposed to various concentrations of scopoletin (30, 100, 300 μ M) for 20 h. Next, 20 μ l of MTT solution (5 mg/ml) was added and the cells were continuously cultured for an additional 4 h. The supernatants were removed and the formazan crystals were dissolved in DMSO. The absorbance at 550 nm was measured by a Model 1500 Multiskan spectrum microplate Reader (Thermo, Waltham, MA, USA).

2.7. Measurement of cytokine levels

RAW 264.7 cells (1×10^6 cells/ml) were seeded and incubated for 2 h in 6-well microplates. Then, cells were treated with various concentrations of scopoletin and MSU (1 mg/ml) for 24 h. The supernatants

were collected and the concentrations of IL-1 β , TNF- α and IL-6 were quantified by the enzyme-linked immunosorbent assay (ELISA) kits (Biosource, Camarillo, CA, USA) according to the manufacturer's instructions.

2.8. Determination of PGE₂ concentration

RAW 264.7 cells were incubated for 24 h with various concentrations of scopoletin and MSU (1 mg/ml). The culture supernatants were collected to determine the concentration of PGE₂ using a direct radio-immunoassay (RIA) kit (Blood Laboratory, Soochow University, Suzhou, China).

2.9. Measurement of NO production

The nitrite level in the medium was measured according to the Griess reaction and the calculated concentration was taken as an indicator of NO production. The culture supernatants were collected as before and mixed with equal volume of Griess reagent (2% sulfanilamide in 5% phosphoric acid and 0.2% naphthylenediamine) and then incubated for 10 min. The absorbance at 540 nm was measured and then calculated against a sodium nitrite standard curve.

2.10. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from RAW 264.7 cells after treatment with a variety of concentrations of scopoletin and MSU (1 mg/ml) using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, the reverse transcription was performed using an oligo(dT)₁₈ primer and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

The PCR reaction was conducted using the following primers (synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd., China). β -actin: sense primer 5'-ACA TCT GCT GGA AGG TGG AC-3', antisense primer 5'-GGT ACC ACC ATG TAC CCA GG-3'; IL-1 β : sense primer 5'-CCT CCA GGA AGG AGC AAA AC-3', antisense primer 5'-TCC TGA GAG AGA GGC AAG AGG A-3'; TNF- α : sense primer 5'-ATG GAT CCA CCA TGA GCA CAG AAA GC-3', antisense primer 5'-ATG AAT TCT CAC AGA GCA ATG ACT CC-3'; IL-6: sense primer 5'-TGC CTT CTT GGG ACT GAT-3', antisense primer 5'-CTG GCT TTG TCT TTC TTG TT-3'; COX-2 (cyclooxygenase-2): sense primer 5'-CTT ACA ATG CTG ACT ATG GCT AC-3', antisense primer 5'-ACT GAT GCG TGA AGT GCT G-3'; iNOS (inducible nitric oxide synthase): sense primer 5'-CCC CAT CAA GCC CTT TAC TT-3', antisense primer 5'-CAC CTC CTG GTG GTC ACT T-3'. The thermocycling programs consisted of 30 cycles at 94 °C for 45 s, 58 °C for 1 min and 72 °C for 1 min for all. The amplified products were electrophoresed on 1.5% agarose gel and visualized by GoldView™ staining (SBS Genetech, Beijing, China) under UV transmission.

2.11. Western blot analysis

RAW 264.7 cells were treated with a variety of concentrations of scopoletin for 20 h and then stimulated with MSU (1 mg/ml) for 20 min. The cells were washed twice with ice-cold phosphate buffered saline (PBS), subsequently the lysis buffer [50 mM Tris-Cl (pH8.0), 150 mM NaCl, 0.02% NaN₃, 1% NP40, 100 μ g/ml PMSF] was added and incubated for 15 min at 4 °C. Then the cell lysates were harvested and centrifuged at 12,000 g for 10 min to remove debris. The protein concentration was determined using Bradford assay. Equal amounts of total proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Following blocking of non-specific binding sites with 5% skim milk in PBST (PBS-tween-20) for 1 h, the membranes were incubated overnight at 4 °C with appropriate dilutions of primary antibodies for detecting I κ B α , phosphor-specific-p65 (serine 276 and

536), ERK1/2, phosphor-ERK1/2, JNK, phosphor-JNK, p38, phosphor-p38 and GAPDH (KangChen Bio-tech, Shanghai, China) and phosphor-I κ B α , phosphor-IKK α antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then they were washed in PBST for three times and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Finally the bands were visualized with the enhanced chemiluminescence (ECL) reagents (KangChen Bio-tech, Shanghai, China) and exposed to X-ray film.

2.12. Statistical analysis

All values were presented as mean \pm S.E.M. Data sets were examined by one way analysis of variance (ANOVA), each group was compared with the control group and determined by Student's unpaired *t*-test. *p*<0.05 and *p*<0.01 were considered significant.

3. Results

3.1. Effect of scopoletin on MSU crystal-induced leukocyte accumulation into murine air pouch

During an acute gout flare, synovial fluid analysis reveals leukocytosis with a high percentage of neutrophil [33]. In order to investigate the acute inflammatory response induced by MSU crystals, subcutaneous air pouches were stimulated with the crystals (3 mg) and the accumulation of inflammatory cells in the exudate was determined. As illustrated in Table 1, the number of infiltrating cells was substantially increased by about 18-fold after six hours of crystal stimulation, and the infiltrating cells were 91% neutrophils and 8% mononuclear cells. Administration of prednisolone (10 mg/kg) via an i.p. injection 30 min before MSU crystals stimulation was able to significantly reduce the total leukocyte infiltration as well as neutrophil and monocyte/macrophage infiltration. Scopoletin at the doses of 100 and 200 mg/kg also remarkably suppressed cellular accumulation. At the highest dose, the reduction percentages are 63.9%, 62.9%, and 58.3%, respectively, suggesting that scopoletin exerted almost equivalent potency with prednisolone, the most commonly used corticosteroids in the treatment of acute gout flare. Our preliminary experiments determined the leukocyte count rose from 0 to 6 h and then subsided, returning close to baseline by 48 h (data not shown).

3.2. Effect of scopoletin on MPO levels in pouch exudates from MSU crystal-induced murine air pouch model

MPO is a hemoprotein that is abundantly expressed in neutrophils (also found in monocytes and some macrophages), and secreted during their activation. It is always taken as an indicator of neutrophil accumulation. MPO possesses potent pro-inflammatory properties and may contribute directly to tissue injury [34]. Therefore, we investigated the

Table 1

Effect of intraperitoneal injection of scopoletin (50, 100, 200 mg/kg) and prednisolone (10 mg/kg) on cell infiltration, including total leukocytes, neutrophils, and monocytes/macrophages in pouch exudates from MSU crystal-induced mouse air-pouch model.

Group	Dose (mg/kg)	Pouch exudate		
		Leukocyte ($\times 10^6$)	Neutrophil ($\times 10^6$)	Monocyte/macrophage ($\times 10^6$)
Normal	–	0.78 \pm 0.15**	0.68 \pm 0.11**	0.16 \pm 0.03**
Control	–	14.39 \pm 1.80	12.93 \pm 1.30	1.20 \pm 0.14
Prednisolone	10	4.38 \pm 0.91**	4.04 \pm 0.89**	0.40 \pm 0.06**
Scopoletin	50	12.21 \pm 1.38	11.04 \pm 1.21	1.08 \pm 0.12
	100	9.83 \pm 1.13*	9.25 \pm 1.04*	0.85 \pm 0.08*
	200	5.19 \pm 0.86**	4.79 \pm 0.88**	0.50 \pm 0.07**

Scopoletin and prednisolone were administered 30 min before the injection of MSU (3 mg) into the air pouch, and pouch exudate was collected 6 h after the stimulation. Data are presented as means \pm S.E.M. from eight mice in each group. **p*<0.05, ***p*<0.01, significantly different from the control group.

MPO levels in the pouch fluid. As depicted in Fig. 2, MPO activity was significantly elevated at 6 h after MSU crystal stimulation. Prednisolone (10 mg/kg) treatment dramatically suppressed the elevation, and scopoletin attenuated MPO activity in a dose-dependent manner.

3.3. Effect of scopoletin on the viability of RAW 264.7 cells

To exclude the possibility that effects on the activated macrophages were due to cytotoxicity, we tested the effects of scopoletin on survival of RAW 264.7 cells using MTT assay. The results showed that scopoletin-induced cytotoxicity was negligible under the concentration of 300 μ M (data not shown).

3.4. Effects of scopoletin on MSU crystal-induced production of pro-inflammatory cytokines and mRNA expression in RAW 264.7 cells

Acute gout flare is an inflammatory process caused by tissue deposition of MSU crystals. Once MSU crystals form and participate, there is an initial interaction with mononuclear cells, which results in the release of pro-inflammatory cytokines as well as other inflammatory mediators, leading to neutrophil recruitment and activation, thus an intense inflammatory response is amplified [31]. Monocytes/macrophages that encounter MSU crystals express, and release pro-inflammatory cytokines including IL-1 β , TNF- α and IL-6 [25–27]. Data of the present study showed that *in vitro* stimulation with MSU crystals dramatically enhanced the production of IL-1 β , TNF- α and IL-6 and the mRNA expression in RAW 264.7 cells. Scopoletin (30, 100, 300 μ M) treatment produced a marked decrease in the elevated levels of IL-1 β and TNF- α (Fig. 3A, B). And it showed an extraordinary inhibition on the production of IL-6, in fact, scopoletin at 300 μ M almost reversed the increased level of IL-6 (Fig. 3C). Moreover, MSU crystal-induced mRNA expression of IL-1 β , TNF- α and IL-6 was suppressed by scopoletin especially at the highest concentration (Fig. 3D–G). These findings demonstrated that scopoletin inhibited the production of pro-inflammatory cytokines through the suppression of their transcription factors.

3.5. Effects of scopoletin on MSU crystal-induced PGE₂ and NO production as well as COX-2 and iNOS mRNA expression in RAW 264.7 cells

Prostaglandins are found in the synovial fluid of gouty arthritis patients, and recent studies revealed that synovial tissues obtained from the patients displayed enhanced expression of iNOS [29,35]. *In vitro*, MSU crystals can increase both COX-2 and iNOS expression in monocyte–macrophages [28,29]. Our investigation further demonstrated this. As shown in Fig. 4, MSU crystal stimulation resulted in a significant promotion of NO and PGE₂ production as well as iNOS and COX-2 mRNA expression in RAW 264.7 cells. Scopoletin (30, 100, 300 μ M)

treatment dramatically inhibited the production of PGE₂ and NO (Fig. 4A, B), and down-regulated the expression of COX-2 and iNOS mRNA (Fig. 4C–E). Our preliminary data indicated that when given at increasing concentrations of MSU (0.05 mg/ml to 2 mg/ml), NO production after 24 h stimulation increased until it reaches the highest level at 1 mg/ml and then subsided. Hence, MSU at 1 mg/ml was used to test the anti-inflammatory effects of scopoletin *in vitro*.

3.6. Effects of scopoletin on MSU crystal-induced activation of NF- κ B in RAW 264.7 cells

NF- κ B regulates the expression of a wide variety of genes that play critical roles in inflammatory responses. These NF- κ B target genes include those encoding cytokines (e.g., IL-1 β , TNF- α , IL-6) and inducible enzymes (e.g., iNOS and COX-2) [36]. Since p65 is the major component of NF- κ B activation, we investigated two phosphorylation sites, ser-276 that is located within the N-terminal Rel homology domain (RHD) and ser-536 that is found within the C-terminal transactivation domain, both of them are essential for the transactivation [37]. Scopoletin significantly inhibited the phosphorylation of p65-ser-276 but had no effect on the phosphorylation of p65-ser-536 (Fig. 5A–C). Ser-276 is phosphorylated exclusively within the cytoplasm by the catalytic subunit of protein kinase A (PKAc), and PKAc is combined to and inactivated by I κ B α , thus the stimulus-induced degradation of I κ B α is prerequisite. Moreover, the canonical p65/p50 heterodimer is largely, though not exclusively, found bound to I κ B α and the nuclear translocation is preceded by phosphorylation and subsequent degradation of I κ B α which was mediated by IKK complex including IKK α . The results presented in Fig. 5A, D, and E demonstrated that scopoletin predominantly suppressed both the phosphorylation and degradation of I κ B α . And the highest concentration showed suppression on the phosphorylation of IKK α as depicted in Fig. 5A, F. These results indicated that scopoletin suppressed the activation of the NF- κ B pathway.

3.7. Effects of scopoletin on MSU crystal-induced phosphorylation of MAPKs in RAW 264.7 cells

The three MAPK molecules (mainly including ERK1/2, JNK and p38) also play an important role by triggering a cascade reaction and ultimately resulting in expression of specific cellular genes encoding pro-inflammatory mediators [38]. Furthermore, they are considered to be the upstream kinases for NF- κ B [37]. As shown in Fig. 6, stimulation with MSU crystal led to evident phosphorylation of MAPK molecules in RAW 264.7 cells. Scopoletin (30, 100, 300 μ M) concentration-dependently inhibited the phosphorylation of ERK1/2 and JNK (Fig. 6A–C) and it showed an outstanding inhibition on p38 activation (Fig. 6A, D). The amount of non-phosphorylated MAPKs was unaffected by scopoletin.

4. Discussion

Gout is an inflammatory arthritis triggered by the crystallization of MSU within the joints in individuals with elevated serum uric acid. Current treatment of gout associated with hyperuricemia entails the uses of anti-inflammatory agents to relieve the symptoms as well as xanthine oxidase inhibitors or uricosuric drugs to lower the serum uric acid. Such a therapy strategy based on drug combinations is generally effective, whereas limitations lie on the adverse events and high economic load. It would be attractive and promising for gout therapy if one compound has both hypouricemic and anti-inflammatory activities.

Previously, our research group found that scopoletin exhibited significant hypouricemic activity as well as remarkable anti-inflammatory activity [16,17], suggesting that scopoletin might be a potential remedy for the treatment of gout. In the current study, we further determined the anti-inflammatory effect of scopoletin in acute gouty arthritis model. To elucidate the underlying mechanism, the influences of scopoletin on

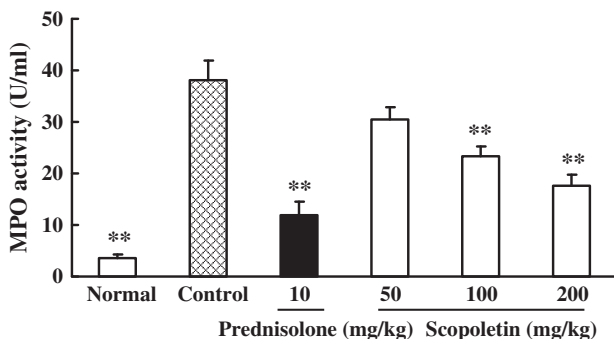


Fig. 2. Effect of scopoletin and prednisolone on elevated MPO activity induced by MSU crystal in mouse air pouch exudate. Scopoletin and prednisolone were administered 30 min before the injection of MSU (3 mg) into the air pouch and pouch exudates were collected 6 h after the stimulation. Data were presented as means \pm S.E.M. from eight mice in each group. * p < 0.05, ** p < 0.01, significantly different from the control group.

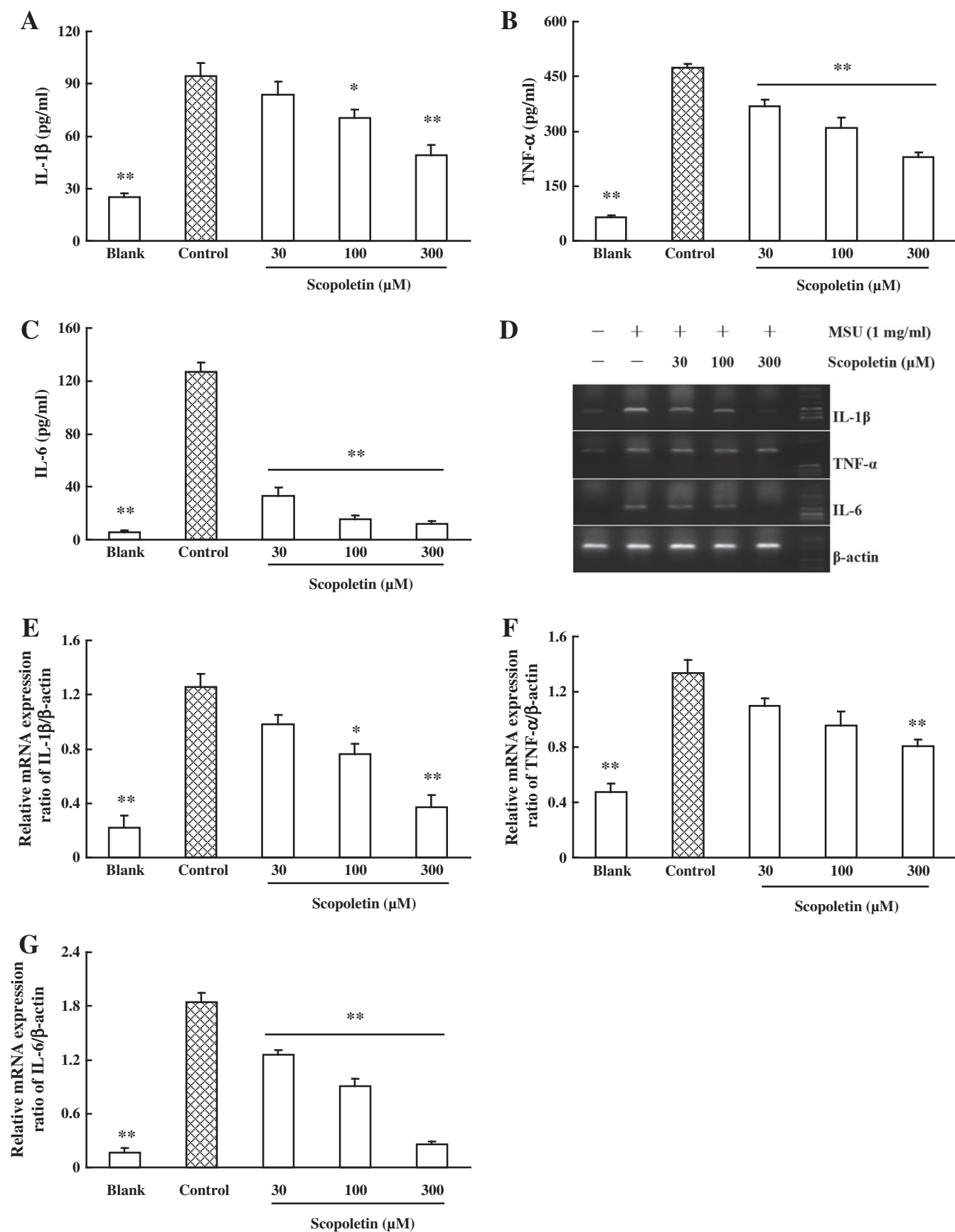


Fig. 3. Effect of scopoletin on MSU crystal-induced production of pro-inflammatory cytokines and mRNA expression in RAW 264.7 cells. The cells were treated with various concentrations of scopoletin in the presence or absence of MSU crystal for 24 h. IL-1 β , TNF- α , and IL-6 levels in the supernatants were measured with ELISA methods (A–C). The mRNA expressions of IL-1 β , TNF- α , IL-6 were measured with RT-PCR analysis (D–G). Blank: un-stimulated group. Control: MSU crystal-stimulated group. Each column represented the mean \pm S.E.M. from three independent experiments. * p <0.05, ** p <0.01, significantly different from the MSU crystal-stimulated group.

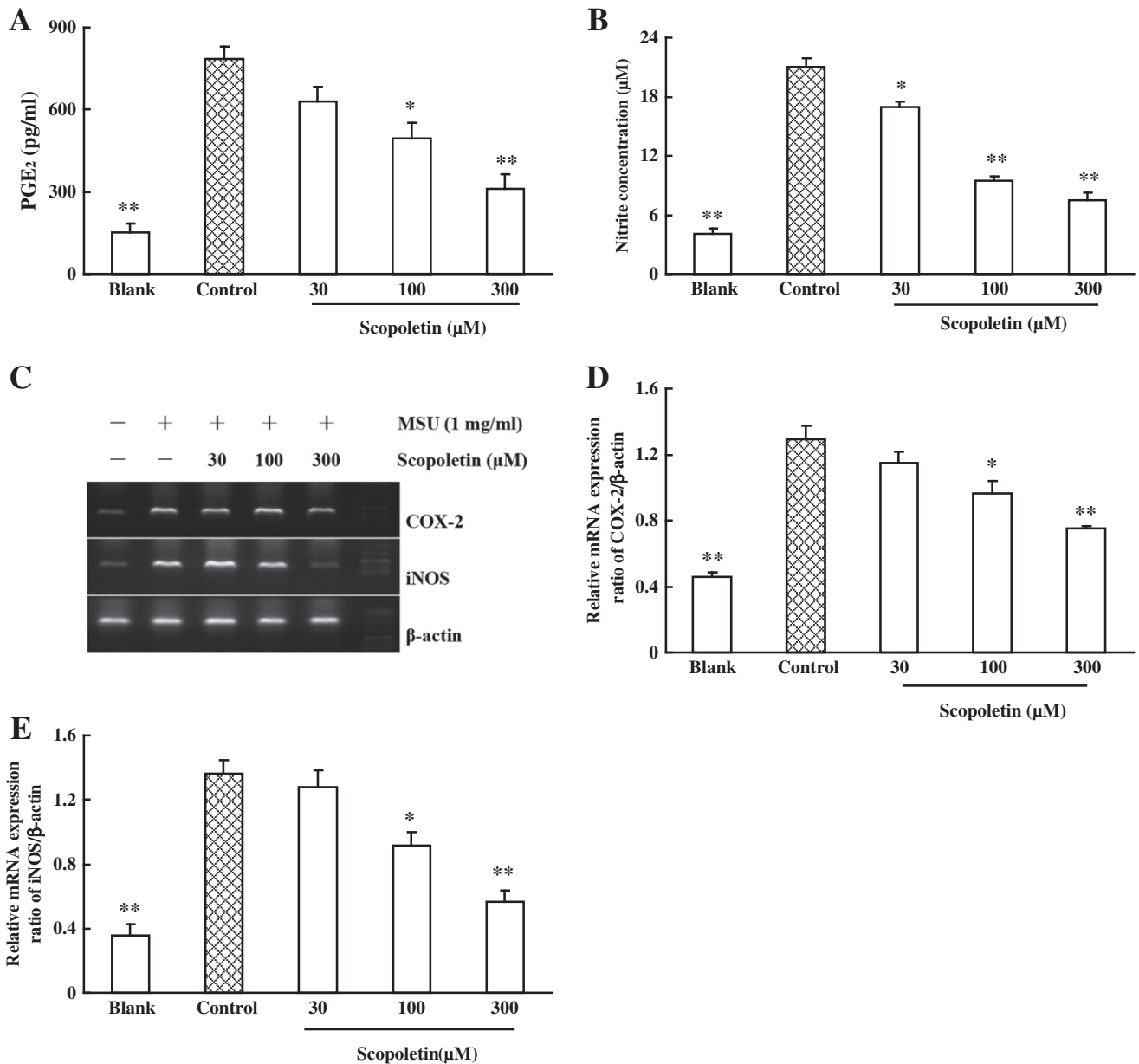


Fig. 4. Effect of scopoletin on MSU crystal-induced PGE₂ and NO production as well as COX-2 and iNOS mRNA expression in RAW 264.7 cells. The cells were treated with various concentrations of scopoletin in the presence or absence of MSU for 24 h. PGE₂ production was measured with the RIA method (A). Nitrite concentration production assay was carried out using Griess reaction as described (B). The COX-2 mRNA expression (C) and the iNOS mRNA expression (D) were measured with RT-PCR method. Blank: un-stimulated group. Control: MSU crystal-stimulated group. Each column represented the mean ± S.E.M. from three independent experiments. **p* < 0.05, ***p* < 0.01, significantly different from the MSU crystal-stimulated group.

MSU crystal-activated macrophages were investigated. Our data has shown for the first time that scopoletin could attenuate MSU crystal-induced acute inflammation in murine air pouch model and inhibit the activation of macrophages *in vitro*.

The MSU air pouch is a well-established model for studying gout, which is built on air pouch model that is induced by subcutaneous injection of air into mice or rats. The 6-day development of the air pouch leads to a pouch lining consisting mainly of mononuclear cells, fibroblasts, mast cells and small blood vessels. This membrane resembles the synovial membrane histologically, and has important properties of the synovial lining. The air pouch model is a very reproducible way of studying the cellular components of inflammation and it has been extensively used for the study of rheumatic disease for over 30 years [32,39]. In the gout air pouch model, injection of MSU crystals into the air pouch evokes infiltration and differentiation of leukocytes, the characteristic features of acute gouty arthritis. In this study, we investigated

the action of scopoletin on the MSU air pouch model in mice. Scopoletin at the doses of 100 and 200 mg/kg remarkably suppressed the total leukocyte accumulation as well as neutrophil and monocyte/macrophage infiltration induced by MSU crystals (Table 1). MPO, as an indicator of neutrophil accumulation, was also determined. MPO also possesses potent pro-inflammatory properties and may contribute directly to tissue injury [34]. Most recent research found that urate can be oxidized by MPO and converted to urate radicals which have considerable potential to influence oxidative stress during inflammation [40]. As depicted in Fig. 2, scopoletin attenuated MPO activity in a dose-dependent manner. Our *in vivo* study presented that scopoletin could inhibit both the leukocyte infusion and the MPO levels that are released from the neutrophils.

Infiltration of vast numbers of neutrophils into inflamed joints is the hallmark of acute gouty arthritis. However, cellular kinetic analyses using rat air pouch model of gout indicate that an increase in monocyte/

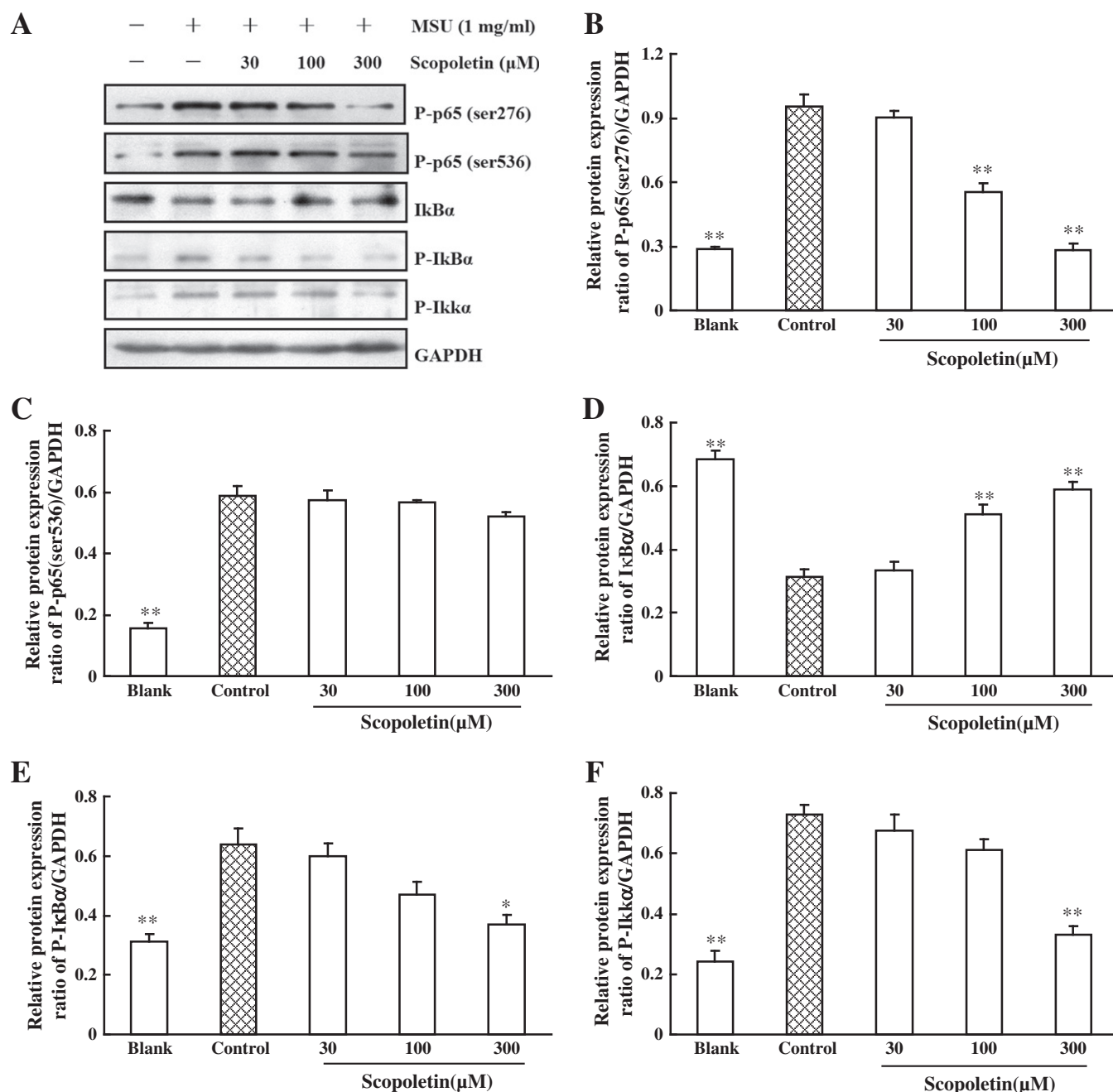


Fig. 5. Effect of scopoletin on NF-κB activation in MSU crystal-activated RAW 264.7 cells. The cells were treated with scopoletin for 20 h and then stimulated with MSU crystal for 20 min. Cellular proteins were prepared and analyzed for P-p65 (ser-276), P-p65 (ser-536), IκBα, P-IκBα, and P-IκKα with western blot. Blank: un-stimulated group. Control: MSU crystal-stimulated group. Each column represented the mean \pm S.E.M. from three independent experiments. * $p < 0.05$, ** $p < 0.01$, significantly different from the MSU crystal-stimulated group.

macrophage density precedes the neutrophil influx [22]. MSU stimulation of the inflammatory cascade is initiated by the activation of articular resident mononuclear phagocytes, leading to a host of pro-inflammatory mediators such as IL-1 β , TNF- α , IL-6, PGE $_2$ and NO [25–29], and resulting in recruitment and ingress of large numbers of neutrophils into the inflamed joints and the amplification of vigorous immune responses. Recent evidence derived from both experimental and clinical studies indicates IL-1 β as a major player in gout [41,42]. As a potent pro-inflammatory cytokine, IL-1 β affects every cell type. It can activate endothelial cells, fibroblasts, lymphocytes, and chondrocytes, and promote infusion of leukocytes [31]. IL-1 β activity could also explain the fever that may be associated with gout flare [43]. Besides IL-1 β , both TNF- α and IL-6 play important roles in the pathogenesis of gout, and they contribute to the acute phase response, endothelial adhesion and

leucocyte accumulation as well [26,27]. Inflammatory prostaglandins (especially the vasoactive PGE $_2$) appear to play a prominent role in the onset of gout attacks because non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit PG formation, are the usual first line treatment for acute gout. Early vasodilation, enhanced vascular permeability, erythema and pain in gouty arthritis are likely to be mediated, at least in part, by PGE $_2$. Excessive, prolonged production of NO also contributes to tissue damage and it has been identified as one of the pro-inflammatory mediators of arthritis in clinical and experimental studies [44]. Expression of iNOS is also increased in the synovial tissue of gouty arthritis patients, suggesting a potential role of NO in gout development [29]. According to the anti-inflammatory effect evaluation, scopoletin did show obvious inhibitory effects on the leukocyte influx. To address whether this effect was owing to the inhibition of MSU-

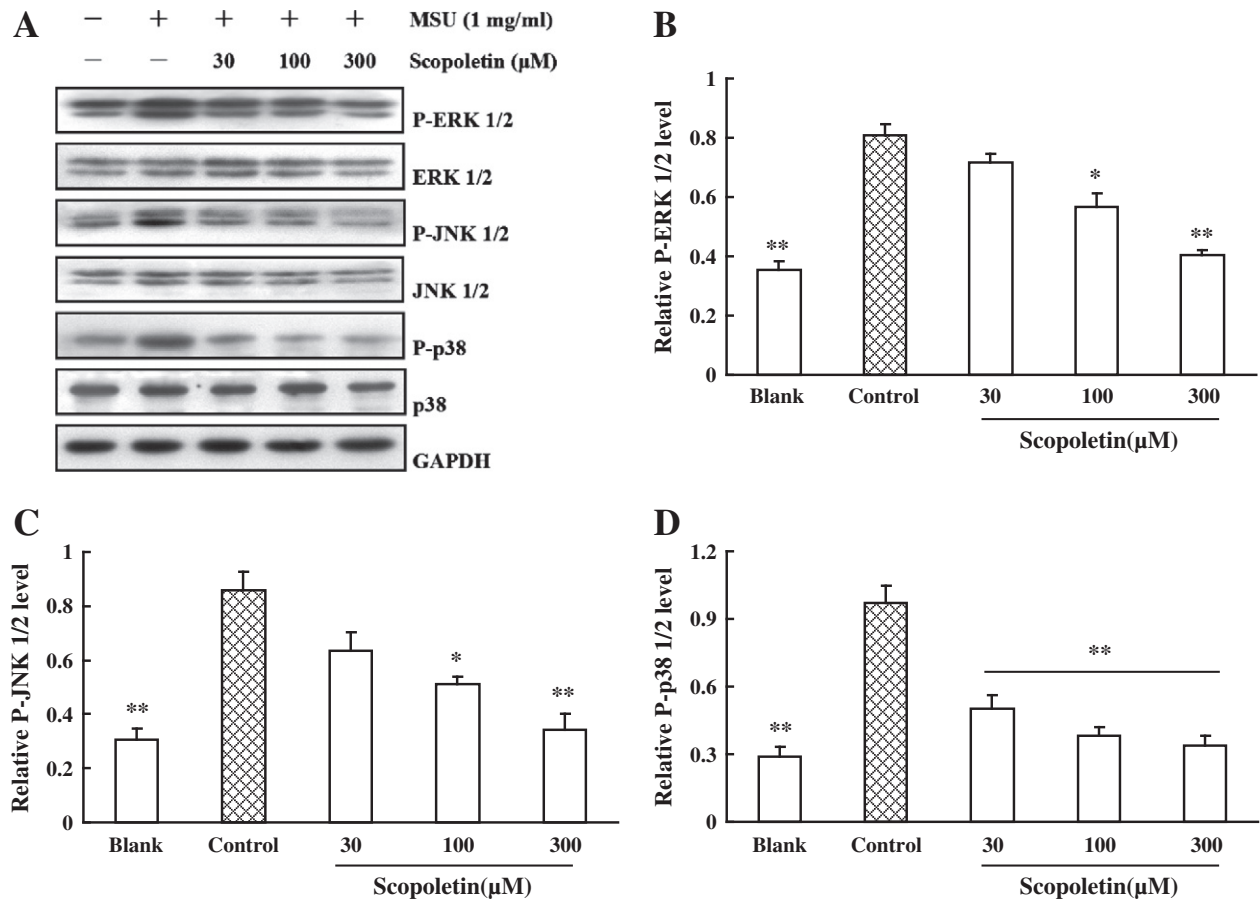


Fig. 6. Effect of scopoletin on ERK1/2, JNK and p38 phosphorylations in MSU crystal-activated RAW 264.7 cells. The cells were treated with scopoletin for 20 h and then stimulated with MSU crystal for 20 min. Cellular proteins were prepared and analyzed for ERK1/2, P-ERK1/2, JNK, P-JNK, p38, and P-p38, respectively. Blank: un-stimulated group. Control: MSU crystal-stimulated group. Each column represented the mean \pm S.E.M. from three independent experiments. * $p < 0.05$, ** $p < 0.01$, significantly different from the MSU crystal-stimulated group.

activated macrophages which take a prominent part in the initiation of the inflammatory cascade, we investigated the effects of scopoletin on the secretion of pro-inflammatory mediators using RAW 264.7 cells, a more mature but not totally differentiated macrophage cell line [45]. The data demonstrated that scopoletin repressed the production of IL-1 β , TNF- α , IL-6, PGE₂ and NO from RAW 264.7 cells to different degrees both at protein and mRNA levels.

It was clear that MSU crystal is the etiological agent of gout, and could induce inflammation in joints since the early 1960s, but the exact underlying pathogenic mechanisms are studied extensively these years. Recent studies found that the pathogenic MSU crystal can act as endogenous danger signal analogous to a motif, called danger-associated molecular pattern (DAMP) that stimulates the innate immune system. Specific membrane-bound TLRs (including TLR2 and TLR4), the large family belonging to pattern recognition receptors (PPRs), were reported to be responsible for the recognition of naked MSU crystals. And TLR adapter protein myeloid differentiation factor 88 (MyD88) is a key signaling molecule in MSU-stimulated inflammation [46]. Once MyD88 is localized to a TLR it recruits other molecules that culminate in NF- κ B signaling and MAPK pathway including ERK, JNK and p38. These signaling pathways lead to the induction of many genes encoding inflammatory mediators such as cytokines, COX-2 and iNOS [36]. Results of the present study implied that scopoletin suppressed the NF- κ B activation through preventing MSU-induced phosphorylation of IKK α and then inhibiting the phosphorylation and degradation of I κ B α and the subsequent phosphorylation of p65-ser-276. At the same time, scopoletin inhibited the phosphorylation of ERK1/2, JNK and p38, which may act alone or relate to the activation of NF- κ B. It was postulated that scopoletin down-regulated gene

transcription and production of the pro-inflammatory mediators possibly by preventing the activation of the canonical NF- κ B pathway and the phosphorylation of MAPK.

In conclusion, the data from the murine model of acute gout flare and the *in vitro* assays indicated that scopoletin could suppress the leukocyte infiltration and activation induced by MSU crystals through the inhibition of the synthesis and release of inflammatory mediators from activated macrophages. Scopoletin might exert anti-inflammatory effects by preventing NF- κ B signaling and MAPK pathway. Whether scopoletin could affect other aspects of the pathogenesis of gout should be further investigated.

In combination with our published studies, we provided four explanations for the potential benefits of scopoletin in gout therapy. The first is hypouricemic activity, aiming to reduce serum urate level. The second is preventing synovial angiogenesis and the third is inducing apoptosis of fibroblast-like synoviocytes, these two merits favor the local environment of joint and prevent further damage in chronic arthritis. The last but the most important is the anti-inflammatory effect in acute gout attack that is demonstrated for the first time in this paper. Because the acute flare is painful and its recurrence rate is high, it may occur even during the urate lowering treatment for long-term prophylaxis. Together with other beneficial effects, it does suggest that the use of scopoletin may provide a safe and effective treatment option for both acute gout and chronic gout.

Conflict of interest

There is no conflict of interest.

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