# Uric acid induces the expression of TNF-α via the ROS-MAPK-NF-κB signaling pathway in rat vascular smooth muscle cells

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**Abstract.** Hyperuricemia and artery atherosclerosis are closely associated and, as a classic inflammatory biomarker, tumor necrosis factor-α (TNF-α) has a direct role in atherogenesis. In the present study, it was demonstrated that uric acid was capable of inducing the generation of TNF-α in vascular smooth muscle cells (VSMCs). The expression levels of proteins were detected using enzyme-linked immunosorbent assays and western blot analysis. The expression levels of mRNAs were determined using reverse transcription-quantitative polymerase chain reaction analysis, and superoxide anion levels were detected using a fluorescence microscope. From the results, it was concluded that uric acid induced the expression of TNF-α in the VSMCs. The antioxidant, N-acetylcysteine, eliminated the uric acid-induced expression of TNF-α. In addition, uric acid increased the level of reactive oxygen species (ROS) and activated the phosphorylation of p38. Subsequent experiments confirmed that the p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580, and nuclear factor (NF)-κB inhibitor, pyrrolidine dithiocarbamate, eliminated the uric acid-induced expression of TNF-α. It was demonstrated that uric acid induced the expression of TNF-α via the ROS-MAPK-NF-κB signaling pathway in VSMCs, providing novel evidence supporting the pro-inflammatory and pro-atherosclerotic effects of uric acid.

## Introduction

Inflammation is involved in the pathological process of several cardiovascular diseases, including atherosclerosis. The results of clinical and basic studies have shown that hyperlipidemia can promote progressive narrowing of the artery lumen (1,2), and increasing epidemiological analyses and experimental data from previous studies support atherosclerosis as a chronic inflammatory disease (3-7). Inflammation is important in the whole process of atherosclerosis.

As an inflammatory biomarker, tumor necrosis factor-α (TNF-α) is an established pro-atherosclerotic factor (8). TNF-α promotes atherosclerosis by increasing the transcytosis of low density lipoprotein (LDL) across endothelial cells and facilitating the retention of LDL in vascular walls (9). TNF-α is capable of inducing the expression of ephrin A1 in a nuclear factor (NF)-κB-dependent manner (10) and activating endothelial cells, which facilitates the promotion of monocyte adhesion to endothelial cells (11-13).

The role of vascular smooth muscle cells (VSMCs) requires consideration in the process of plaque formation and the progression of atherosclerosis. The local inflammatory factors produced by VSMCs may have a direct and essential effect on the process of atherosclerosis and the development of cardiovascular complications.

The association between increased serum uric acid and cardiovascular risk, including hypertension or coronary artery disease, in the general population has been demonstrated (14-16). It has been reported that uric acid is able to stimulate VSMC proliferation and oxidative stress via the vascular renin-angiotensin system (17) in addition to increasing the expression of platelet-derived growth factor A-chain (18). Uric acid is also a marker of chronic inflammation, and can cause the secretion of inflammatory factors *in vitro* and *in vivo* (19-21); it can induce the expression of C-reactive protein in VSMCs (22). This evidence suggests that uric acid is capable of accelerating the generation and progression of atherosclerosis via the pro-inflammatory response in the vessel wall.

Although uric acid is known to evoke responses of inflammation *in vitro* and *in vivo*, there is no direct evidence to indicate that the pro-inflammatory effect of uric acid on VSMCs is through TNF-α. Therefore, the present study examined the effect of uric acid on the expression of TNF-α and its mechanism in VSMCs, with a particular focus on the reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways.

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## Materials and methods

*Reagents*. Dulbecco's high-glucose modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Uric acid, PD98059, SB203580, SP600125, N-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC), thenoyltrifluoroacetone (TTFA) and diphenyleneiodonium (DPI) were produced by Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Rabbit polyclonal TNF-α antibody was provided by Invitrogen; Thermo Fisher Scientific, Inc. Mouse GAPDH antibody was ordered from CoWin Biotech Co., Ltd. (Beijing, China). Phosphorylated-p38 antibody, p38 antibody and 2',7'-dichlorodihydrofluororescein diacetate ($H_2$DCF-DA) were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Relative secondary antibodies were provided by CoWin Biotech Co., Ltd. (Beijing, China), and the enzyme-linked immunosorbent assay (ELISA) kit for detecting TNF-α was from West Tang Biotechnology (Shanghai, China).

*Culture of rat VSMCs*. VSMCs were isolated from the thoracic aorta of male Sprague-Dawley rats according to a previous report (23). Prior to the experiment, rats (n=8, 6 weeks old, 150-180 g, were purchased from the Institutional Animal Care Committee of Xi'an Jiaotong University, Xi'An, China) had free access to food and water, and were maintained in a constant environment with a conventional 12/12 h light/dark cycle. Isolated VSMCs were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere of 5% $CO_2$, 95% air at 37˚C. After 7 days, the cells exhibited a typical 'hill and valley' growth morphology. The cells between passages 3 and 10 were used for all experiments. Prior to the experiments, the cells were incubated in serum-free medium for an additional 24 h. All experimental procedures were performed in accordance with the international, national and institutional guidelines, and approved by the Central Hospital of Hanzhong (Hanzhong, China).

*MTT assay of VSMC viability*. The viability of the VSMCs was assessed using the MTT method. VSMCs (1-1.5x$10^6$) were incubated with uric acid (10-160 mg/l) for 12 h, or with 80 mg/l of uric acid for 0, 3, 6, 12 and 24 h at 37˚C. Subsequently, 20 $\mu$l of MTT (5 mg/ml; Invitrogen; Thermo Fisher Scientific, Inc.) was added to each well. Following incubation at 37˚C for 4 h, the culture medium was removed and the formazan crystals were dissolved by adding 150 $\mu$l DMSO (Sigma-Aldrich; Merck KGaA). Following vigorous agitation for 10 min, the absorbance was measured at 490 nm on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

*ELISA analysis*. The VSMCs were cultured in a 96-well plate and co-cultivated with the indicated agents for the indicated times. Subsequently, the supernatant was collected via centrifugation (1,080 x g, 5 min) at 4˚C and detected for TNF-α using an ELISA kit (Cat: F16960) specific for rat TNF-α.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of the mRNA expression of TNF-e*. Total RNA was purified from the VSMCs using a RNA fast 200 purification kit (Xianfeng Biotech, Shaanxi, China). cDNA was synthesized using a Revert Aid™ First Strand cDNA synthesis kit (Takara Bio, Inc. Tokyo, Japan) according to the manufacturer's protocol. The cDNA (200 ng) was amplified using the following primer pairs (0.25 mM for forward and reverse primers) specific for rat, with GAPDH amplified as an internal control for normalization: GAPDH, sense 5'-GCA AGTTCAACGGCACAGTCAAG-3' and antisense 5'-ACA TACTCAGCACCAGCATCACC-3'); TNF-α, sense 5'-ATG GGCTCCCTCTCATCAGT-3' and antisense 5'-GCTTGG TGGTTTGCTACGAC-3'. The expression of mRNA was expressed as relative to the internal control. The RT-qPCR analysis was performed using the ABI PRISM® 7300 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction products were detected by measuring the binding of SYBR Green I to DNA using the SYBR Green PCR Master Mix (Cat: 04913850001, Roche Diagnostics, Basel, Switzerland). The optimization of the amplification reaction was assured by dissociation curve analysis. The basic protocol for the qPCR was as follows: Initial incubation at 95˚C for 1 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec, and 72˚C for 30 sec. All samples were run in triplicate, and were analyzed using the $2^{-\Delta\Delta Cq}$ method, as previously described (24).

*Measurement of ROS in VSMCs*. VSMCs (1-1.5x$10^6$) were pretreated with NAC ($10^{-2}$ M), TTFA ($10^{-5}$ M) or DPI ($10^{-5}$ M) for 1 h incubation at 37˚C, followed by cultivation with uric acid (80 mg/l) for 3 h. For the positive ROS up control group, cells were pretreated with ROS up (Beyotime Institute of Biotechnology) for 20 min. $H_2$DCF-DA (10 $\mu$M) was then added to each well for 1 h at 37˚C and the cells were subsequently washed with PBS three times. Fluorescence images were captured at the excitation wavelength of 488 nm and the emission wavelength of 525 nm under a fluorescence microscope (Olympus, Tokyo, Japan). The fluorescence intensity of the experimental field was measured and analyzed from the fluorescence images using Image-Pro Plus software (Version 10; Media Cybernetics, Inc., Silver Springs, MD, USA). The relative fluorescence intensity was calculated as the average value from six repeated experiments.

*Western blot analysis*. Following the indicated treatments, the VSMCs were washed with ice-cold PBS three times, and the whole cell lysates were acquired by adding lysis buffer supplemented with protease inhibitor cocktail and phosphatase inhibitors (Roche Diagnostics, Basel, Switzerland). The concentration of protein was measured using a BCA protein assay kit (Bio-Rad Laboratories, Inc.). Equal quantities of protein extract (40 $\mu$g) were loaded and separated on a 10% SDS-PAGE gel, and then transferred onto PVDF membranes (0.22-$\mu$m; EMD Millipore, Billerica, MA, USA). The membranes were then incubated with anti-TNF-α (Cat: 25-7423-82, 1:1,500 dilution), anti-GAPDH (Cat: CW0101 M, 1:2,000 dilution), anti-p38 (Cat: AM076, 1:1,000 dilution) or anti-phosphorylated-p38 (Cat: AM071, 1:500 dilution) antibodies overnight at 4˚C. Following washing, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Goat anti-rabbit, Cat: CW0103S; Goat anti-mouse, Cat: CW0102S) diluted 1:6,000, for 2.5 h at 25˚C, followed

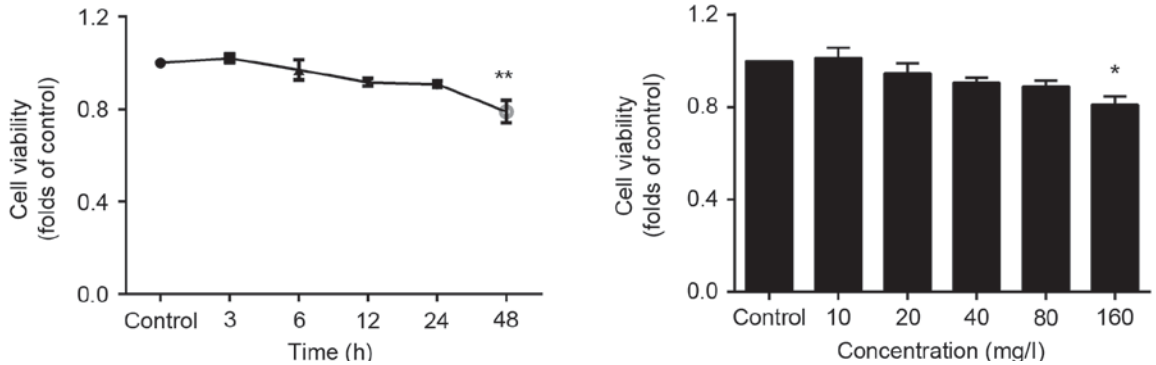


Figure 1. Effect of uric acid on the viability of vascular smooth muscle cells. The cells were co-cultivated with different concentrations of uric acid for 12 h, or with a uric acid concentration of 80 mg/l for 0, 3, 6, 12, 24 and 48 h. Cell viability was then detected using the MTT method. The results are expressed as the mean ± standard error of the mean from six independent experiments. *P<0.05 and **P<0.01, vs. control.

by enhanced chemiluminescence (Cat: WBKLS0050, Merck KGaA).

*Statistical analysis*. All values are presented as the mean ± standard error of the mean. Statistical significance between groups was assessed using one-way analysis of variance, followed by multiple comparisons using GraphPad Prism software (version 6; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

*Effect of uric acid on VSMC viability*. As shown in Fig. 1, treatment with concentrations of uric acid between 10 and 80 mg/l had no significant effect on the viability of the VSMCs. In addition, no significant effect on the viability of the VSMCs was observed when the cells were exposed to the uric acid concentration of 80 mg/l for 3, 6, 12 and 24 h.

*Uric acid induces the expression of TNF-α in VSMCs*. The protein expression levels of TNF-α were detected using ELISA and the mRNA expression levels were detected using RT-qPCR analysis. As shown in Fig. 2A and B, the protein and mRNA expression levels of TNF-α in the VSMCs were significantly increased following exposure to 40 and 80 mg/l uric acid for 12 h (P<0.05 and P<0.01, vs. control) in a concentration-dependent manner. In addition, the results, as shown in Fig. 2C and D, showed that uric acid at a concentration of 80 mg/l increased the protein and mRNA levels of TNF-α in a time-dependent manner.

*Uric acid stimulates the generation of ROS in VSMCs*. To investigate whether ROS are involved in the uric acid-induced expression of TNF-α, the protein expression levels of TNF-α were determined using western blot analysis. As shown in Fig. 3A, the VSMCs co-cultivated with the antioxidant, NAC, eliminated the uric acid-induced expression of TNF-α. The levels of intracellular ROS were further analyzed using the ROS fluorescent probe, $H_2$DCF-DA. As shown in Fig. 3B, minor DCF fluorescence was observed in the control VSMCs, which represented basal ROS generation, whereas uric acid (80 mg/l) increased the generation of ROS in the VSMCs (P<0.01, vs. control). However, pre-incubation of the cells with NAC ($10^{-2}$ M), TTFA ($10^{-5}$ M) and DPI ($10^{-5}$ M) reduced the uric acid-stimulated generation of ROS in the VSMCs (P<0.001, vs. uric acid alone).

*Uric acid induces the expression of TNF-α via the ROS-MAPK-NF-κB signaling pathway*. As it is reported that MAPK and NF-κB are important in the expression of several inflammatory cytokines (25,26), the upregulation of TNF-α by uric acid in VSMCs may be associated with MAPK-NF-κB signaling. The results of the present study showed that the protein expression of TNF-α in VSMCs was increased following stimulation with 80 mg/l for 24 h. Pretreatment of the cells with SB203580 (p38 MAPK inhibitor) or PDTC (NF-κB inhibitor) for 1 h significantly reduced the uric acid-induced expression of TNF-α, however, PD98059 (extracellular signal-regulated kinase 1/2 inhibitor) and SP600125 (Janus kinase inhibitor) did not have this effect (Fig. 4A and B). As the results described above indicated that ROS are involved in the uric acid-induced expression of TNF-α in VSMCs, the present study investigated whether the activation of p38 was mediated by ROS in the VSMCs by determining the levels of phosphorylated p38. The results showed that phosphorylated p38 was markedly increased following stimulation of the cells with uric acid for 1 h. However, NAC and SB203580 significantly inhibited the uric acid-induced phosphorylation of p38 (Fig. 4C).

## Discussion

Uric acid is an end product generated by the metabolism of endogenous and exogenous purines in humans. Several findings have led to the ongoing reappraisal of the role of uric acid in cardiovascular disease (27-29). The findings of previous basic and clinical studies have indicated that uric acid may be an independent risk factor for cardiovascular disease and kidney disease (30). Other studies have reported that an elevated level of uric acid predicts the development of hypertension, obesity, kidney disease and diabetes (31-34). Studies using animal models and cell cultures have identified mechanisms by which uric acid may induce cardiovascular and renal disease. There is increasing evidence that serum uric acid may be crucial in inflammatory responses. Atherosclerosis is a type of cardiovascular disease, and is also

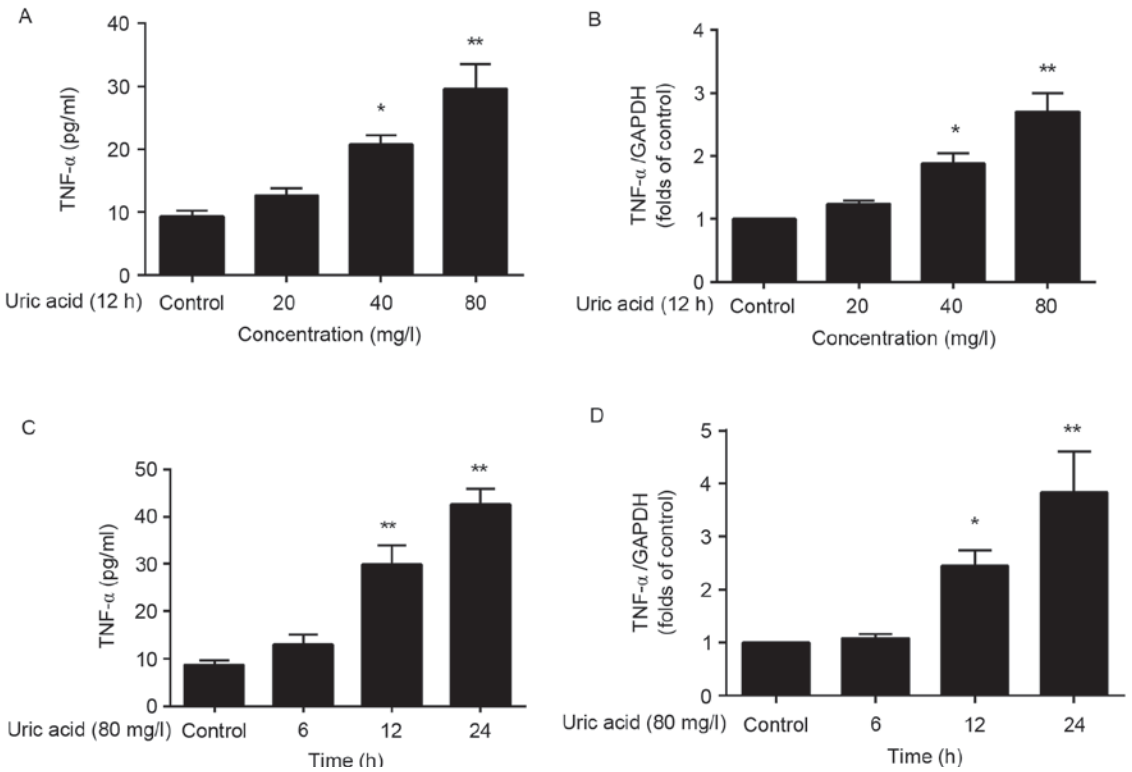


Figure 2. Uric acid induces the protein expression of TNF-α in vascular smooth muscle cells. Concentration-dependent increases in the (A) protein and (B) mRNA expression of TNF-α. Time-dependent increases of the (C) protein and (D) mRNA expression of TNF-α. The cells were treated with 20, 40 or 80 mg/l uric acid for 12 h, or with 80 mg/l uric acid for the indicated durations. The protein expression levels of TNF-α were then identified using ELISA and mRNA levels were determined using RT-qPCR analysis. The results are expressed as the mean ± standard error of the mean from six independent experiments for ELISA and triplicate experiments for RT-qPCR analysis. $^{*}$P<0.05 and $^{**}$P<0.01, vs. control. ELISA, enzyme-linked immunosorbent assay; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TNF-α, tumor necrosis factor-α.

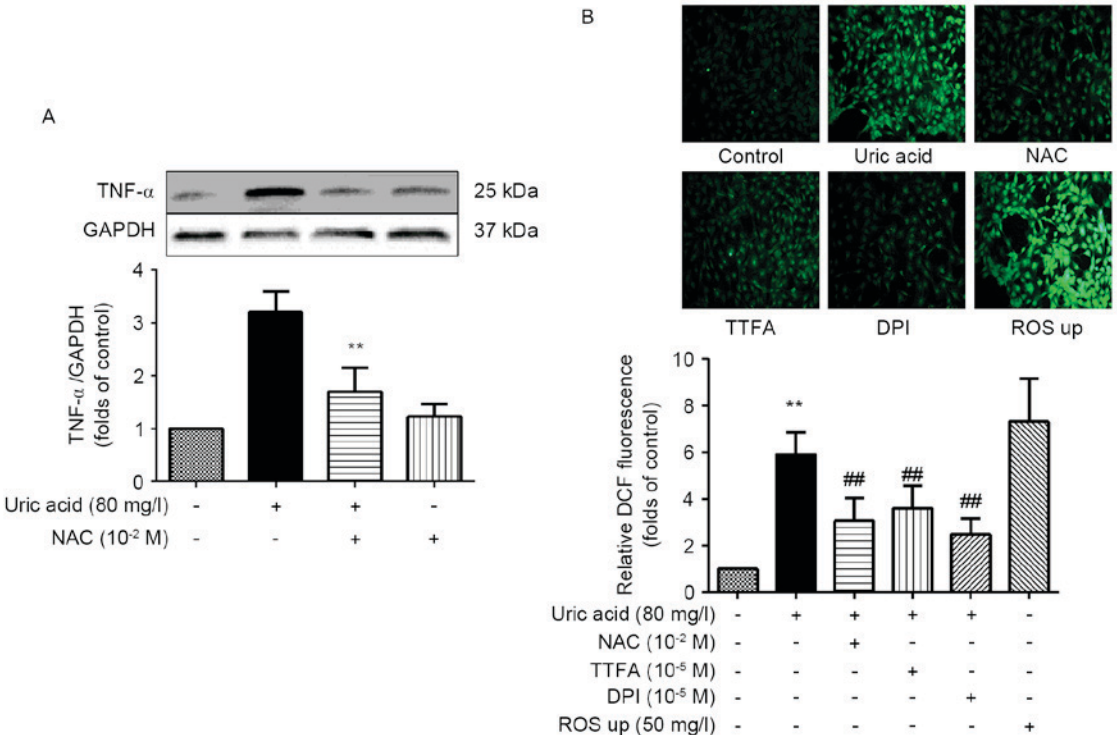


Figure 3. Involvement of the ROS pathway in the uric acid-induced expression of TNF-α and intracellular superoxide anion generation in vascular smooth muscle cells. (A) Cells were pretreated with NAC ($10^{-2}$ M) for 1 h prior to incubation with uric acid (80 mg/l) for 24 h and then examined using western blot analysis. (B) Cells were exposed to uric acid (80 mg/l) for 3 h following pretreatment with NAC ($10^{-2}$ M), TTFA ($10^{-5}$ M) or DPI ($10^{-5}$ M) and detected using a fluorescence microscope (magnification, x200). The results are expressed as the mean ± standard error of the mean from three independent experiments for western blot analysis and six independent experiments for the analysis of ROS. $^{**}$P<0.01, vs. control; $^{\#\#}$P<0.01, vs. uric acid alone. TNF-α, tumor necrosis factor-α; NAC, N-acetylcysteine; TTFA, thenoyltrifluoroacetone; DPI, diphenyleneiodonium; ROS, reactive oxygen species; ROS up, ROS positive control.

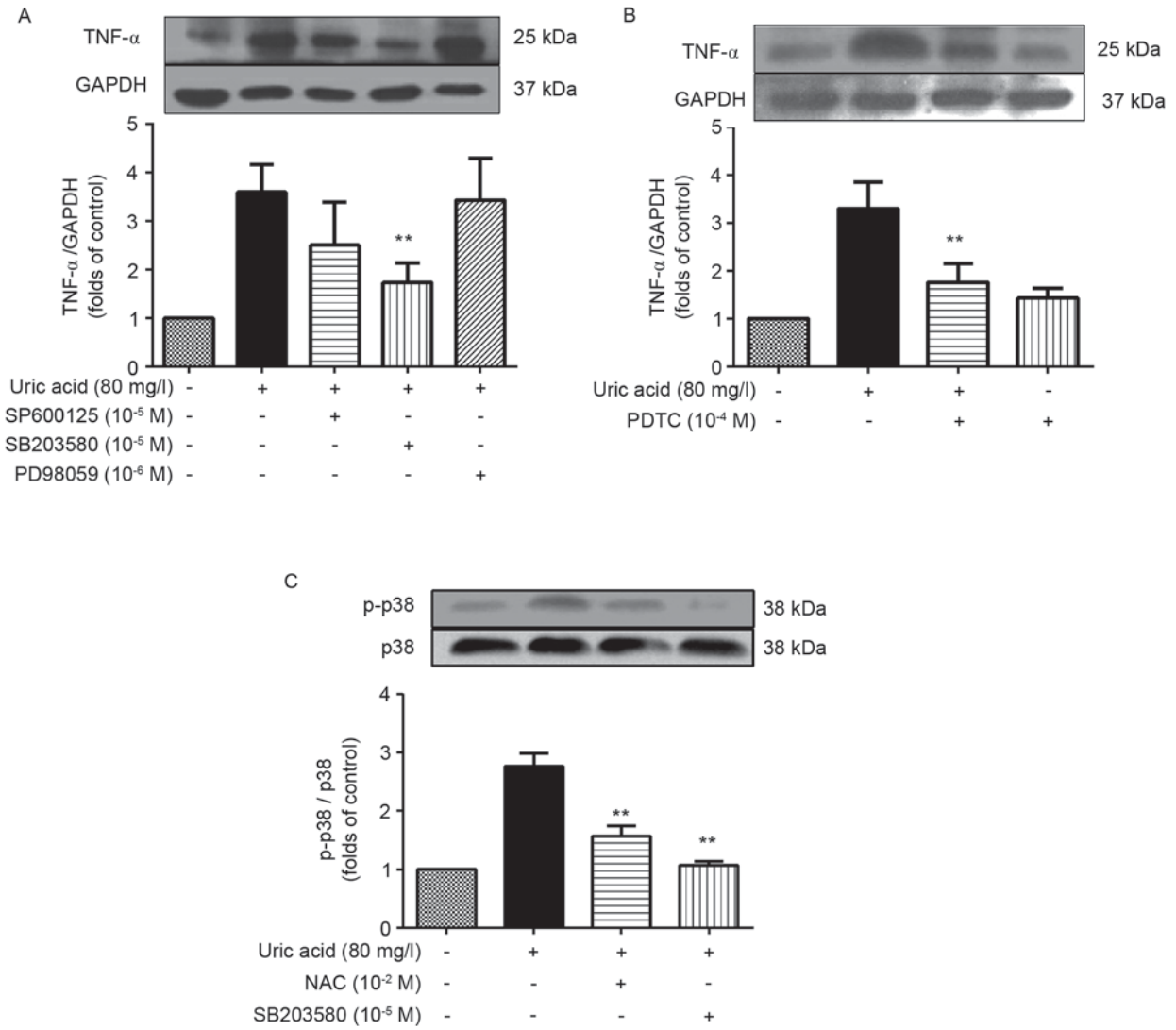


Figure 4. Involvement of the ROS-MAPK-NF-κB signaling pathway in the uric acid-induced expression of TNF-α in vascular smooth muscle cells. (A) Cells were pretreated with SB203580, SP600125, PD98059 or (B) PDTC for 1 h, prior to incubation with uric acid (80 mg/l) for 24 h. The expression of TNF-α was examined using western blot analysis. (C) Levels of p-p38 were detected prior to pretreatment with SB203580 or NAC for 1 h, followed by incubation with uric acid (80 mg/l) for 1.5 h. The results are expressed as the mean ± standard error of the mean from three independent experiments. $^{**}P<0.01$, vs. uric acid alone. ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; NAC, N-acetylcysteine; PDTC, pyrrolidine dithiocarbamate.

a chronic inflammatory disease. Inflammation is important in the progression of atherosclerosis. As a classic inflammatory biomarker, TNF-α is directly involved in atherogenesis. In the present study, it was found that uric acid induced the expression of TNF-α in VSMCs in a time-dependent and concentration-dependent manner.

ROS are important secondary messengers and are directly involved in oxidative stress. A low concentration of uric acid can exert a protective effect against the effect of ROS *in vivo*, whereas a high concentration of uric acid has the opposite effect (35,36). The results of the present study showed that ROS are important in the uric acid-induced expression of TNF-α. Pre-treatment with the antioxidant, NAC ($10^{-2}$ M), significantly inhibited the protein expression of TNF-α in the VSMCs; whereas cells co-cultivated with TTFA (complex II inhibitor) and DPI (NADPH oxidase inhibitor) reduced uric acid-induced superoxide anion generation in the VSMCs.

MAPK and NF-κB signaling are important in inflammation (37-39). The activation of NF-κB is responsible for the expression of several inflammatory cytokines. Soluble uric acid has been found to induce the expression of monocyte chemoattractant protein-1 from VSMCs through the activation of NF-κB and p38 MAPK (17). In the production of pro-inflammatory cytokines (40), a previous *in vivo* experiment demonstrated that uric acid is a potent pro-inflammatory molecule derived from dying cells. The results of the present study showed that p38 MAPK and NF-κB were involved in the expression of TNF-α induced by uric acid; the selective p38 MAPK and NF-κB inhibitor, PDTC, significantly inhibited the expression of TNF-α in the VSMCs.

In conclusion, the present study demonstrated that uric acid induced the expression of TNF-α in VSMCs through the ROS-p38MAPK-NF-κB signaling pathway. These findings provide novel evidence supporting the potential inflammatory effect and pro-atherosclerotic effect of uric acid.

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