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Original Article

The pro-inflammatory effect of uric acid in human umbilical vein endothelial cells via ROS-PI3K/AKT-NF-κB signal pathway

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Abstract: Inflammation plays an important role in the pathological process of many cardiovascular diseases specially atherosclerosis. As a biomarker of inflammatory Tumor necrosis factor-α (TNF-α) plays a key role in the process of atherogenesis. In this research we investigated the pro-inflammatory effect of uric acid in human umbilical vein endothelial cells. Our research indicated that uric acid was able to induce TNF-α expression in HUVECs. Antioxidant NAC abolished uric acid-induced TNF-α expression. In addition, uric acid stimulated generation of reactive oxygen species (ROS) and activated AKT phosphorylation. The further study confirmed that PI3K/AKT inhibitor LY294002 and NF-κB inhibitor pyrrolidine dithiocarbamate both abolished uric acid induced TNF-α expression. In conclusion, uric acid is able to induce TNF-α expression via PI3K/AKT-NF-κB signal pathway in HUVECs, which provides a new evidence for the pro-inflammatory and pro-atherosclerotic effects of uric acid.

Keywords: Uric acid, tumor necrosis factors-α, inflammatory, atherosclerosis

Introduction

Inflammation plays an important role in the pathological process of many cardiovascular diseases such as atherosclerosis [1]. Research suggests that atherosclerosis is a progressive narrowing of the artery lumen due to hyperlipidemia [2-4]. Inflammation plays an important role in the whole process of atherosclerosis [5-8].

Tumor necrosis factor-α (TNF-α) is an established pro-atherosclerotic factor and also act as an inflammation biomarker [9-11]. It plays an important role in the process of atherosclerosis via increasing the low density lipoprotein (LDL) transcytosis across endothelial cells and thereby facilitating LDL retention in vascular walls [12]. TNF-α can up regulate the expression of ephrinA1 by a NF-κB dependent manner and also can activate endothelial cells, so it's easy to promote monocyte adhesion to endothelial cells.

Endothelial cells (ECs) play an important role in the regulation of vascular physiological functions and the maintenance of vascular homeostasis, and endothelial dysfunction is an early step in the development of atherosclerosis [13-15]. Many large epidemiological studies have identified a strong association between increased serum uric acid and cardiovascular risks such as hypertension or coronary artery disease in the general population [16, 17]. This evidences indicated that uric acid is closely related with atherosclerosis. It's reported that uric acid stimulates vascular smooth muscle cell proliferation and oxidative stress via the vascular renin-angiotensin system as well as increasing platelet-derived growth factor A-chain expression [18]. Uric acid is also a maker of chronic inflammation, can also cause the secretion of inflammatory factors both *in vitro* and *in vivo*, such as induce the expression of C-reactive protein in vascular smooth muscle cells [19]. All those suggest that uric acid may accelerate the initiation and progression of atherosclerosis through the pro-inflammatory response in vessel wall. While, there is no direct evidence to demonstrate the pro-inflammatory effect of uric acid on human umbilical vein endothelial cells. Therefore, in this research we

examined the effect of uric acid on TNF-α expression and it's mechanism in human umbilical vein endothelial cells, particularly focus on reactive oxygen species (ROS), phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) and nuclear factor kappaB (NF-κB) signal pathway.

Materials and methods

Reagents

RPMI 1640 Medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Uric acid was from MP Biomedicals (Irvine, CA, USA). LY294002, N-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC), thenoyltrifluoroacetone (TTFA) and diphenyleneiodonium (DPI) were produced by Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal TNF-α antibody was provided by Abnova (Taipei, Taiwan). B-actin antibody was ordered from CoWin Biotech (Beijing, China). AKT antibody and 2',7'-dichlorodihydrofluororescein diacetate (H_2DCF-DA) were obtained from Beyotime (Jiangsu, China). Relative second antibody were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA), ELISA kit for detecting TNF-α was from West tang (Shanghai, China).

Culture of rat HUVECs

HUVECs (from ATCC, Manassas, VA, USA) cultured in RPMI 1640 Medium supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Before the experiments, the cells were incubated in 1% FBS medium for an additional 12 h. All experimental procedures were performed in accordance with the international, national and institutional rulers.

MTT assay of HUVECs viability

The viability of HUVECs was detected by the MTT method. HUVECs were incubated with DMEM supplemented and added uric acid (10-160 mg/L) for 12 h or with the concentration of uric acid 80 mg/L for 0, 3, 6, 12 and 24 h. Then, 20 μL MTT (5 mg/ml, supported by Invitrogen, Carlsbad, CA, USA) was added to each well. After further incubation for 3.5 h, the culture medium was removed and the formazan crystal was dissolved by addition of 150 μL DMSO (Sigma-Aldrich, St. Louis, MO, USA) to each well with vigorous shaking for 10 min. finally, the absorbance was measured at 490 nm with micro plate reader (Bio-Rad, Hercules, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The cells were cultured in 96 well plate and stimulated with the indicated agents for the indicated time. Then, the supernatant was collected and assayed for TNF-α by ELISA kit specific for human TNF-α. The absorbance was measured at 490 nm with micro plate reader (Bio-Rad, Hercules, CA, USA).

Quantitative real-time PCR for TNF-α mRNA expression

Total RNA was purified from HUVECs using RNA fast 100 purification kit according to the instruction of the kit (Xianfeng biotech, shaanxi, China). Equal DNA (cDNA) was synthesized from total RNA by Revert AidTM First Strand cDNA synthesis kit (TaKaRa, Tokyo, Japan) following the manufacturer's instructions. The cDNA was amplified using primer pairs specific for human GAPDH was amplified as an internal control for normalization (sense primer: 5'-CTCCTGTTCGACAGTCAGCC-3', anti-sense primer: 5'-TTCCCGTTCTCAGCCTTGAC-3'); TNF-α (sense primer: 5'-AGCCCATGTTGTAGCAAACC-3', anti-sense primer: 5'-ACATTGGGTCCCCCAGGATA-3'). Expression of mRNA was expressed as relative to internal control. The real-time PCR was performed using the Mx3000P® QPCR System (Agilent Technologies, Santa Clara, CA, USA). The reaction products were detected by measuring the binding of SYBR Green I to DNA using the SYBR Green PCR Master Mix. The optimization of the amplification reaction was assured by a dissociation curve analysis. The basic protocol for real-time PCR was an initial incubation at 94°C for 1 min, followed by 35 cycles of 94°C 18 s, 60°C 30 s, and 72°C 30 s. All samples were run in triplicate, and then analyzed using the $2^{-(\Delta\Delta Ct)}$ methods as previously described.

Measurement of reactive oxygen species in HUVECs

The HUVECs were exposed to uric acid (80 mg/L) for 3 h after pretreatment with NAC (10^{-2} mol/L), TTFA (10^{-5} mol/L), DPI (10^{-5} mol/L) for 2

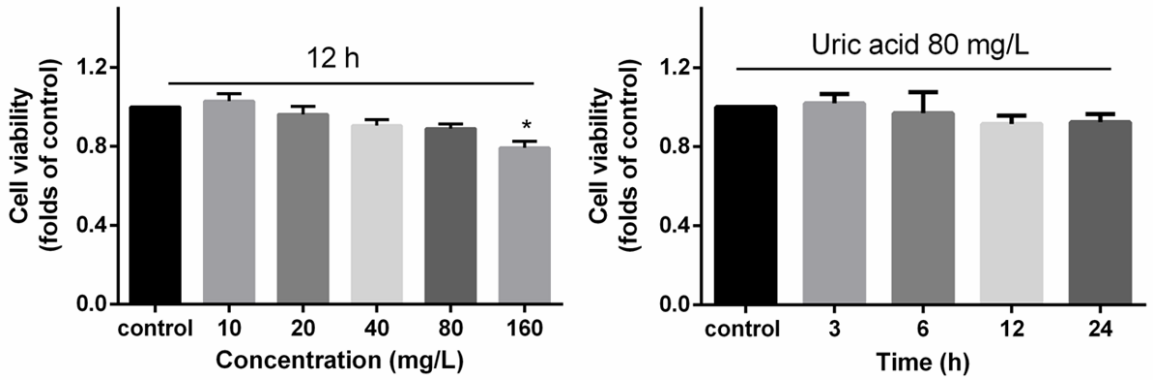

Figure 1. Effect of uric acid on the viability of HUVECs. The cells were incubated with the different concentrations of uric acid for 12 h or with the concentration of uric acid 80 mg/l for 0, 3, 6, 12 and 24 h. Then, the cell viability was assayed by the MTT method. Results were expressed as mean ± S.E.M. from six independent experiments. *; $P < 0.05$ *vs.* control.

h. Then, the cells were loaded with H_2DCF-DA (10 μmol/L) for 1 h and washed with PBS three times. Fluorescence images were acquired at the excitation wavelength of 488 nm and the emission wavelength of 525 nm with fluorescence microscope (Olympus, Tokyo, Japan). Fluorescence intensity of the experimental field was measured and analyzed from the fluorescence images with the Image-pro plus software (Version X; Media Cybernetics, Silver Springs, MD, USA). The relative fluorescence intensity was taken as the average of values from six repeated experiments.

Western blot

The HUVECs were washed with PBS after the treatments. The whole cell lysates were prepared in lysis buffer supplemented with protease inhibitor cocktail and phosphatase inhibitors (Roche, Basel, Switzerland). Equal amount of protein extract (50 μg) was loaded, separated by 10% SDS-PAGE, and blotted onto PVDF membrane (0.45 μm Merck Millipore, Billerica, MA, USA). The membranes were incubated with anti-TNF-α (1:1000 dilution), anti-β-GAPDH (1:3000 dilution) or anti-akt (1:1500 dilution) antibodies overnight at 4°C. After washed, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse second antibody (1:10000 dilution) followed by the enhanced chemiluminescence.

Statistical analysis

All values were shown as means ± SEM. Statistical significance between groups was assessed using one-way ANOVA, followed by post hoc Duncan multiple comparisons with the SPSS (Chicago, IL, USA). A value of $P < 0.05$ was considered to be statistically significant.

Results

The effect of uric acid on HUVECs viability

To explore whether uric acid was able to influence the cell viability of HUVECs in vitro, the cells were subjected to different concentration of uric acid for 12 h or 80 mg/L for different times. **Figure 1** showed that uric acid from 10 mg/L to 80 mg/L did not significantly affect the viability of HUVECs; when exposed to the concentration of uric acid 80 mg/L for 3, 6, 12, 24 h, it's also did not affect the viability of HUVECs.

Uric acid induced TNF-α expression in HUVECs

The TNF-α protein was detected by ELISA and mRNA was tested by Real-time qPCR. As shown in (**Figure 2A**, **2B**) TNF-α protein and mRNA in HUVECs was significantly increased after exposure to 40 or 80 mg/L uric acid for 12 h ($P < 0.05$ or $P < 0.01$ vs. control) and had a concentration-dependent manner. The result in (**Figure 2C**, **2D**) displayed that uric acid at 80 mg/l increased TNF-α protein and mRNA level in a time-dependent manner.

Uric acid stimulated ROS generation in HUVECs

To investigate whether ROS participated in uric acid-induced TNF-α, the expression of TNF-α

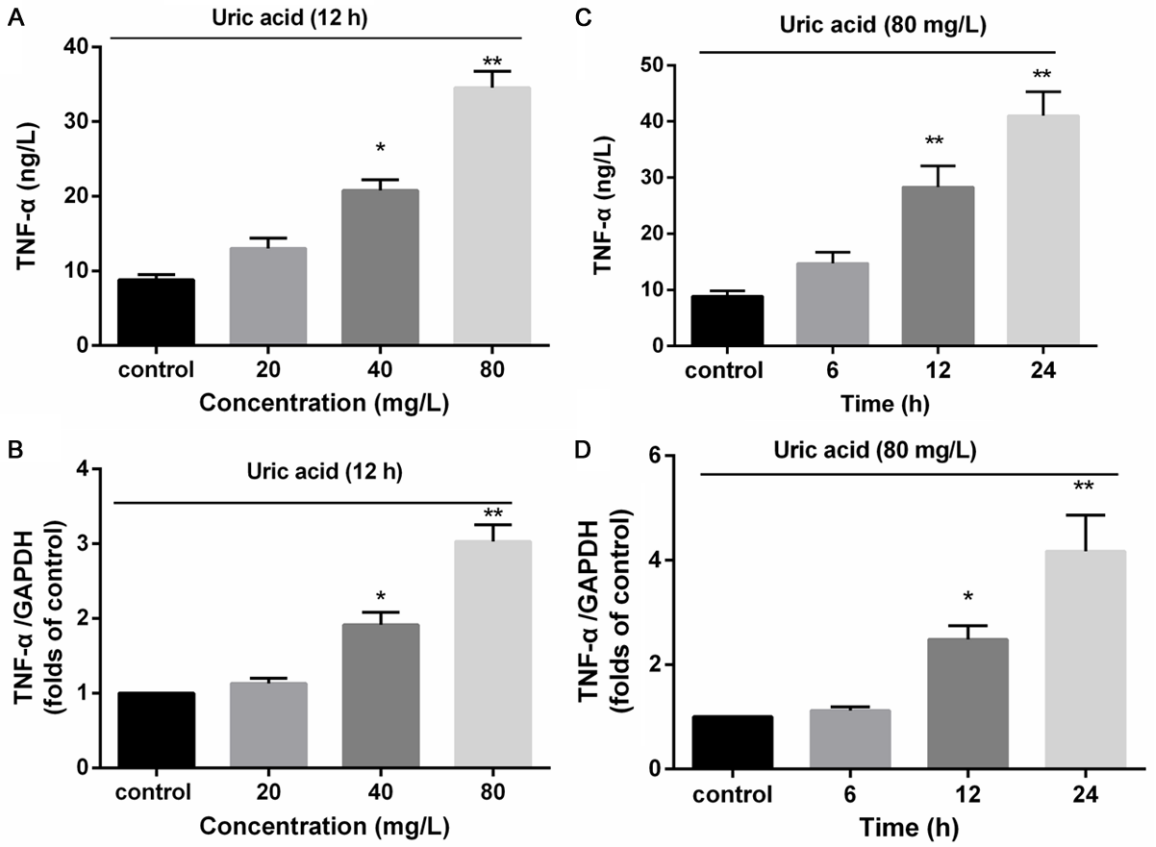

Figure 2. Uric acid induces TNF-α protein expression in HUVECs. A, B. Concentration-dependent increase of TNF-α protein or mRNA expression; C, D. Time-dependent increase of TNF-α protein or mRNA expression. The cells were treated with 20, 40, 80 mg/L uric acid for 12 h or 80 mg/L uric acid for the indicated times. Then, TNF-α protein expression was identified by ELISA and mRNA was tested by Real-time PCR. Results were expressed as mean ± S.E.M. from six independent experiments for ELISA result and triplicate for PCR. *; $P < 0.05$ *vs.* control, **; $P < 0.01$ *vs.* control.

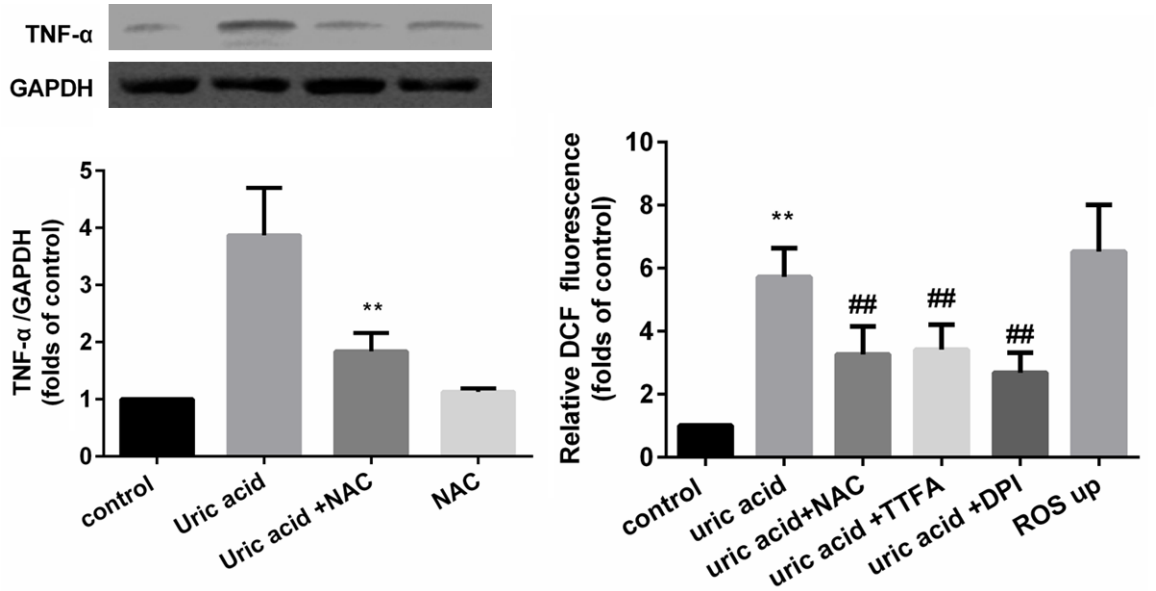

Figure 3. Involvement of ROS pathway in uric acid-induced TNF-α expression and the intracellular superoxide anion generation in HUVECs. The cells were pretreated with NAC (10^{-2} mol/L) for 2 h before incubation with uric acid (80

mg/L) for 24 h and then tested by western blot. The cells were exposed to uric acid (80 mg/L) for 3 hours after pretreatment with NAC (10^{-2} mol/L), TTFA (10^{-5} mol/L), DPI (10^{-5} mol/L) and the detected by fluorescence microscope. Results were expressed as mean ± S.E.M. from three independent experiments for western blot and six independent experiments for the analysis of ROS. **; P < 0.01 *vs.* control, ##; P < 0.01 *vs.* uric acid alone.

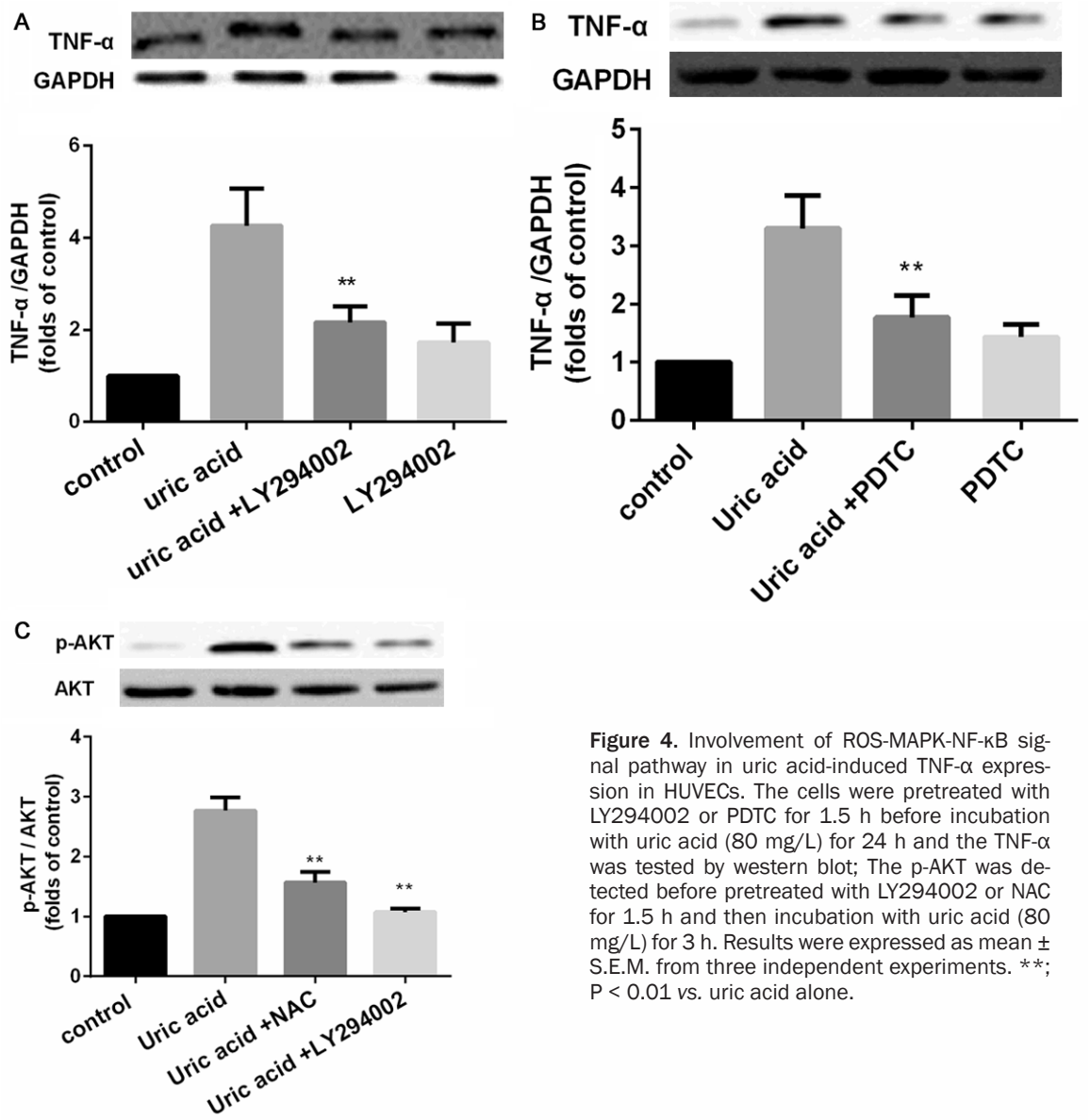

Figure 4. Involvement of ROS-MAPK-NF-κB signal pathway in uric acid-induced TNF-α expression in HUVECs. The cells were pretreated with LY294002 or PDTC for 1.5 h before incubation with uric acid (80 mg/L) for 24 h and the TNF-α was tested by western blot; The p-AKT was detected before pretreated with LY294002 or NAC for 1.5 h and then incubation with uric acid (80 mg/L) for 3 h. Results were expressed as mean ± S.E.M. from three independent experiments. **; P < 0.01 *vs.* uric acid alone.

was performed by western blot. As shown in **Figure 3A** that pretreatment of HUVECs with antioxidant NAC abolished uric acid-induced TNF-α expression. And the intracellular ROS were determined with ROS fluorescent probe H_2DCF-DA. As seen from **Figure 3B**, minor DCF fluorescence in control HUVECs was observed, which represented the basal ROS generation. Uric acid (80 mg/L) increased ROS generation in HUVECs (P < 0.01 vs. control). However, pre-incubation of the cells with NAC (10^{-2} mol/L), TTFA (10^{-5} mol/L), DPI (10^{-5} mol/L) reduced uric acid-stimulated ROS generation in HUVECs (P < 0.001 vs. uric acid alone).

Uric acid induces TNF-α expression via ROS-PI3K/AKT-NF-κB signal pathway

PI3K/AKT and NF-κB participate in the expression of many inflammatory cytokines and in this

experiment we explore whether the expression of TNF-α in HUVECs was related to PI3K/AKT-NF-κB signaling. The results showed that after the stimulation with 80 mg/L for 24 h, the expression of TNF-α protein was increased in HUVECs. While, pretreatment of the cells with LY294002 (PI3K/AKT inhibitor) or PDTC (NF-κB inhibitor) for 1.5 h significantly reduced uric acid-induced TNF-α expression (**Figure 4A**, **4B**). The previous results indicated that ROS was involved in Uric acid-induced TNF-α expression in HUVECs. To probe whether ROS mediated uric acid induced AKT activation in HUVECs, phosphorylated AKT was determined. The results displayed that phosphorylated AKT was obviously increased after stimulation of the cells with uric acid for 3 h. However, pretreatment of HUVECs with NAC and LY294002 for 1.5 h prior to exposure to uric acid significantly inhibited uric acid induced AKT phosphorylation (**Figure 4C**).

Discussion

Uric acid is an end product generated by the metabolism of endogenous and exogenous purine in humans [20]. Several events have led to the ongoing reappraisal of the role of uric acid in cardiovascular disease [21-23]. Some studies that have controlled for multiple risk factors indicated that uric acid may be an independent risk factor for both cardiovascular disease and kidney disease. Other studies have noted that an elevated level of uric acid predicts the development of hypertension, obesity, and diabetes [24-27]. Studies using animal models and cell cultures have identified mechanisms by which uric acid might induce cardiovascular and renal disease. There is growing evidence that serum uric acid might play a crucial role in inflammatory responses [28]. Atherosclerosis is a kind of cardiovascular, and also a chronic inflammatory disease and endothelial dysfunction is an early event in atherosclerosis that precedes clinical symptoms and has prognostic value for future cardiovascular events. Inflammation plays an important role in the progress of atherosclerosis. As a classic inflammatory biomarker TNF-α plays a direct role in atherogenesis [29, 30]. In this study we have found that uric acid can induce TNF-α expression in HUVECs and which has a time-dependent or concentration-dependent manner.

Reactive oxygen species (ROS) are both the important second messenger and the direct participant of oxidative stress. Low concentration of uric acid can exit a protect effect from the impact of ROS in vivo, while high concentration of uric acid have an opposite effect [31]. The recent researches show that ROS play the important role in the uric acid elicited the expression of TNF-α. Pro-treatment with antioxidant NAC 10^{-2} M can significantly inhibit the expression of TNF-α protein in HUVECs; while pretreatment of cells with TTFA (complex II inhibitor) and DPI (NADPH oxidase inhibitor) can reduce uric acid-induced superoxide anion generation in HUVECs.

The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway has been shown to play an important role in inflammatory responses in vitro and in vivo [32]. While, NF-κB plays a prominent role in transcriptional regulation of most inflammatory genes that contribute to the development of the expressions of many inflammatory cytokines [33]. Soluble uric acid has been found to induce monocyte chemo attractant protein-1 (MCP-1) from vascular smooth muscle cells through activation of NF-κB and p38 mitogen-activated protein kinase (MAPK) [19]. In vivo experiment demonstrated that uric acid has a potent ability as a pro-inflammatory molecule derived from dying cells [34]. Our result exhibited that PI3K/AKT and NF-κB was involved in the expression of TNF-α induced by uric acid, since the selective PI3K/AKT LY294002 and NF-κB inhibitor PDTC significantly blocked the expression of TNF-α in HUVECs.

Conclusions

The present study demonstrates that uric acid induced the expression of TNF-α in HUVECs by ROS-PI3K/AKT-NF-κB signal pathway. These provide the new evidence for the potential inflammatory effect and pro-atherosclerotic of uric acid.

Disclosure of conflict of interest

None.

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