# Original Article Potential risk of hyperuricemia: leading cardiomyocyte hypertrophy by inducing autophagy

Xiao-Jie Zhang<sup>1\*</sup>, Dong-Mei Liu<sup>1\*</sup>, Ying Sun<sup>1</sup>, Yan-Shan Li<sup>2</sup>, Li-Li Ma<sup>1</sup>, Xiu-Fang Kong<sup>1</sup>, Xiao-Meng Cui<sup>1</sup>, Rong-Yi Chen<sup>1</sup>, Zhuo-Jun Zhang<sup>1</sup>, Lin-Di Jiang<sup>1,3</sup>

<sup>1</sup>Department of Rheumatology, Zhongshan Hospital, Fudan University, Shanghai 200030, China; <sup>2</sup>Department of Rheumatology, Linyi People's Hospital, Linyi 276000, Shandong, China; <sup>3</sup>Center of Clinical Epidemiology and Evidence-based Medicine, Fudan University, Shanghai 200030, China. <sup>\*</sup>Equal contributors.

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**Abstract:** Background: Clinical studies have shown that hyperuricemia is associated with many cardiovascular diseases; however, the mechanisms involved remain unclear. In this study, we investigated the effect of uric acid on cardiomyocytes and the underlying mechanism. Methods and results: H9c2 cardiomyocytes were treated with various concentrations of uric acid. 3-Methyladenine (3-MA) or Compound C was added before treatment with uric acid. The expression of myocardial hypertrophy-related genes was measured using polymerase chain reaction (PCR). The cell surface area was calculated using ImageJ Software. Western blotting was used to measure the protein levels. Uric acid increased the gene expression of *Nppa*, *Nppb*, and *Myh5*, which are involved in myocardial hypertrophy, and the relative cell surface area of cardiomyocytes in a dose-dependent manner. Consistently, the ratio of LC3II/I, which is a biomarker of autophagy, increased dose-dependently, whereas the protein level of p62, a protein that is degraded by autophagy, decreased. 3-MA, an autophagy inhibitor, rescued uric acid-induced myocardial hypertrophy. Treatment with uric acid increased the level of phosphorylated adenosine monophosphate kinase (AMPK), as well as its downstream effector unc-51-like kinase (ULK1). Pharmacological inhibition of AMPK by Compound C attenuated the uric acid-induced activation of autophagy and myocardial hypertrophy. Conclusions: Uric acid induces myocardial hypertrophy by activating autophagy via the AMPK-ULK1 signaling pathway. Decreasing the serum uric acid level may therefore be clinically beneficial in alleviating cardiac hypertrophy.

Keywords: Uric acid, cardiomyocyte hypertrophy, autophagy, AMPK, ULK1

#### Introduction

Uric acid is the end product of purine metabolism in humans. Hyperuricemia is caused by overproduction of uric acid in the liver, reduced excretion of urate by the kidneys or a combination of the two [1]. In addition to acting as an important antioxidant within the circulation [2], uric acid may also function as a pro-oxidant within cells, which is detrimental to organisms [3]. Clinically, an elevated serum uric acid has been reported to be positively associated with various cardiovascular diseases and conditions, including heart failure, myocardial infarction, stroke, atrial fibrillation, and cardiovascular mortality [4-9].

The decreased viability and the increased apoptosis of cardiomyocytes upon exposure to

uric acid may partly contribute to uric acid-related cardiovascular diseases [10, 11]. In addition, compromised insulin signaling and insulin resistance in cardiomyocytes following uric acid treatment may also explain the increased risk of cardiovascular diseases [12]. Furthermore, the level of voltage-gated potassium channel subfamily 1 number 5 (Kv1.5) is stabilized by uric acid, which may result in hyperuricemia-associated atrial fibrillation [13]. However, upon uric acid treatment, the cellular hypertrophic changes remain unclear.

Using three-dimensional speckle tracking echocardiography, the left ventricular mass index, which is an indicator of left ventricular hypertrophy, was found to be increased in subjects with hyperuricemia compared with that of healthy subjects. A higher left ventricular mass index was also observed in individuals with both hyperuricemia and hypertension compared to those with hypertension alone [14]. Specifically, the left ventricular mass index was positively correlated with the serum uric acid level [14]. These findings point to an underlying hypertrophic effect of uric acid on cardiomyocytes. Thus, in the current study, the hypertrophic effect of uric acid on cardiomyocytes was investigated *in vitro*.

Upon uric acid treatment, the levels of various intracellular enzymes are changed. For example, adenosine monophosphate kinase (AMPK) is an enzyme that is regulated by uric acid [15-17]. As a central metabolic sensor that governs energy metabolism in response to alterations in nutrients and intracellular energy levels [18], the enzymatic activity of AMPK was either upregulated or downregulated upon uric acid treatment in hepatocytes, pancreatic  $\beta$  cells, and macrophages [15, 16]. However, whether the activity of AMPK is regulated by uric acid in cardiomyocytes is not known.

Among the downstream substrates of AMPK, ULK1 is activated and phosphorylated by AMPK at Ser317, Ser467, Ser555, Thr574, Ser637, and Ser777 [19]. Activation of ULK1 initiates the formation of autophagosomes, which play a pathogenic role in cardiac hypertrophy [20]. Thus, in the present study, we focused on the effects of soluble uric acid on myocardial hypertrophy and further explored the possible involvement of the AMPK-ULK1autophagy signaling pathway in uric acid-induced myocardial hypertrophy. We report herein the potential risk of hyperuricemia, which leads to cardiomyocyte hypertrophy through activation of autophagy by the AMPK-ULK1 signaling pathway.

#### Materials and methods

#### Reagents

Uric acid, 3-methyladenine (3-MA), Compound C, and TRIzol reagent were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Anti-AMPK, anti-phospho-AMPK $\alpha$  (Thr172), anti-ULK1, and anti-phospho-ULK1 (Ser555) antibodies were obtained from Cell Signaling Technologies (Beverly, MA, USA). Anti-LC3B, anti-p62, and anti- $\beta$ -actin antibodies were pro-

cured from Abcam (Cambridge, UK). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS; Australian origin), and penicillinstreptomycin were purchased from Gibco (Grand Island, NY, USA). The BCA protein assay kit, radioimmunoprecipitation assay lysis buffer, phenylmethanesulfonyl fluoride (PMSF), and hematoxylin-eosin were obtained from Bevotime Biotechnology (Shanghai, China). Phos-STOP was procured from Roche (Basel, Switzerland). The PrimeScript RT reagent kit (Perfect Real Time) was purchased from Takara Biotechnology (Tokoyo, Japan). The PCR primers were synthesized by Sangon Biotechnology (Shanghai, China). SYBR Green dye was obtained from Yeasen Biotechnology Co. (Shanghai, China).

# Preparation of soluble uric acid

NaOH solvent was prepared by dissolving 400 mg NaOH in 10 ml ddH<sub>2</sub>O, then 150 mg uric acid was added to the solvent and dissolved at 55°C. Different concentrations of soluble uric acid were diluted with DMEM. The pH of the solution was adjusted by adding HEPES or carbon dioxide.

# Cell culture and treatment

The in vitro experiments were conducted with H9c2 cardiomyocytes (Shanghai Cell Bank of the Chinese Academy of Sciences, Shanghai, China). The cells were cultured in DMEM supplemented with 10% FBS, 1% HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. To assess myocardial hypertrophy, the cells were exposed to various concentrations of uric acid (0, 5, 10, 15, or 20 mg/dl) for 48 hours upon reaching 60-70% confluence. In experiments with inhibitors, the cells were pretreated with 3-MA (5 mM), an autophagy inhibitor, or Compound C (5 µM), an inhibitor of AMPK, for 1 hour before coincubation with 20 mg/dl uric acid.

#### Measurement of the cell surface

The cells were fixed for 15 min, stained with hematoxylin-eosin for 1 min, visualized, and photographed under a microscope (Olympus Corp., Waltham, MA, USA). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to calculate the cell surface area.

#### RNA extraction and real-time PCR analyses

Total RNA was extracted with TRIzol reagent and then reverse transcribed into cDNA using the PrimeScript<sup>TM</sup> RT reagent kit (Perfect Real Time) (TaKaRa Biomedical Technology Co., Ltd., Beijing, China). Real-time PCR was performed using SYBR Green dye with an Applied Biosystems 7500 Real-Time System 9 (Applied Biosystems, Foster City, CA, USA). Relative gene expression was normalized to Gapdh by using the  $\Delta\Delta$ CT method.

#### Western blotting

The cells were harvested and lysed with radioimmunoprecipitation assay lysis buffer supplemented with phosSTOP solution and 1 mM PMSF. The protein concentration was determined using a BCA protein assay kit. Equal amounts of protein were loaded onto an 8% or 12% SDS-polyacrylamide gel and run at 80 V for 30 min, followed by 120 V for 60-90 min. Subsequently, the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Burlington, MA, USA), which was subsequently blocked with 5% skim milk for 1 hour at room temperature. The membrane was incubated with a primary antibody overnight at 4°C and then washed three times with Tris-buffered saline-Tween 20 (TBST). The membrane was then incubated with a secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature. After washing the membrane with TBST, the immunoreactive signals were acquired by enhanced chemiluminescence with a Tanon-5200-Multi System (Tanon Co. Shanghai, China), and the protein bands were quantified using ImageJ software with normalization to β-actin.

#### Statistical analysis

All statistical analyses were conducted using SPSS 22.0 (IBM, Armonk, NY, USA). Experiments using cells were repeated independently at least three times. The data are presented as the mean  $\pm$  standard deviation (SD). Differences among the groups were determined by one-way analysis of variance (ANOVA). A *p*-value < 0.05 was considered statistically significant.

#### Results

Uric acid induces myocardial hypertrophy in a dose-dependent manner

Following exposure to uric acid, there was a significant increase in the mRNA expression of natriuretic peptide type A (*Nppa*), natriuretic peptide type B (*Nppb*), and myosin heavy chain 7 (*Myh7*), which are biomarkers of myocardial hypertrophy, and their mRNA levels increased in a dose-dependent manner (**Figure 1A**).

To confirm the hypertrophic response to uric acid, the cell surface area was examined. The cell surface area increased significantly following treatment with 15 mg/dl or 20 mg/dl uric acid (**Figure 1B, 1C**). These results indicate that soluble uric acid induces myocardial hypertrophy.

#### Uric acid induces autophagy in a dose-dependent manner

Although normal autophagy is essential for cellular homeostasis under physiological conditions, pathological autophagy is associated with cardiomyocyte hypertrophy [21-23]. To investigate whether autophagy participates in uric acid-induced myocardial hypertrophy, the levels of autophagic biomarkers were measured. The expression of autophagy-related gene 5 (Atg5), which is essential for the formation of autophagic vesicles, was upregulated in a dose-dependent manner following uric acid treatment (Figure 2A). Furthermore, the level of LC3II, which is a biomarker that reflects the degree of autophagy, was increased dosedependently, whereas the level of p62, a protein that is degraded by autophagy, was decreased (Figure 2B, 2C). These results indicate that autophagy was induced upon uric acid treatment in a dose-dependent manner.

# Uric acid activates myocardial hypertrophy by triggering the autophagy pathway

To determine whether uric acid-induced myocardial hypertrophy is mediated by the autophagy activation, 3-MA, an inhibitor of autophagy, was used before treatment with uric acid to block autophagy. Treatment with 3-MA attenuated the uric acid-induced increase in the mRNA expression of *Nppa*, *Nppb*, and *Myh7* (**Figure 3A**). Consistently, the cell surface area was reduced to a level comparable to that of

# Uric acid and cardiomyocyte hypertrophy



**Figure 1.** Soluble uric acid induces H9c2 cardiomyocyte hypertrophy. H9c2 cardiomyocytes were treated with different concentrations of uric acid. A. Real-time PCR analysis of the mRNA expression of the hypertrophy-related genes *Nppa*, *Nppb* and *Myh7* after uric acid exposure. B. Cells were hematoxylin-eosin stained and visualized with a microscope after uric acid exposure. Bars indicate for 20  $\mu$ m. C. And the cell surface areas were measured using ImageJ software. The data are expressed as mean  $\pm$  SD; \*P < 0.05 and \*\*P < 0.01 indicate significant differences compared to the control group.

the control (**Figure 3B**, **3C**). These data reveal that uric acid-induced myocardial hypertrophy is mediated by autophagy activation.

#### Uric acid activates the AMPK-ULK1 pathway

AMPK, a key protein that is involved in the regulation of autophagy [20], is regulated by uric acid [16]. To investigate whether uric acid treatment was accompanied by AMPK-ULK1 pathway activation, western blotting was performed at the indicated times. The phosphorylation of AMPK at Thr172 and ULK1 at Ser555 was significantly increased with prolonged exposure of cells to uric acid (**Figure 4A, 4B**).

#### The AMPK-ULK1 pathway is involved in the induction of myocardial hypertrophy through autophagy

To explore whether uric acid-induced autophagy was mediated by the AMPK-ULK1 pathway, Compound C was used to inhibit AMPK activity. The uric acid-induced increased expression of *Atg5* was attenuated by preincubation with Compound C (**Figure 5A**). Furthermore, the increased LC3-II/LC3-I ratio and decreased p62 level were also normalized (**Figure 5B**, **5C**). Correspondingly, *Nppa*, *Nppb*, and *Myh7* expression was reduced (**Figure 5D**), and the cell surface area decreased to a level comparable to that of the control (**Figure 5E**, **5F**).

#### Discussion

In the current study, we found that uric acid treatment induced myocardial hypertrophy, and the hypertrophic effect was enhanced with increasing concentrations of uric acid. We also found that autophagy mediated uric acidinduced myocardial hypertrophy. The AMPK-ULK1 signaling pathway was responsible for the activation of autophagy and subsequent myocardial hypertrophy upon uric acid treatment.

Here, we found that cardiomyocytes treated with soluble uric acid underwent hypertrophic

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**Figure 2.** Soluble uric acid induces H9c2 cardiomyocyte autophagy. H9c2 cardiomyocytes were treated with different concentrations of uric acid. A. Real-time PCR analysis of the mRNA expression of the autophagy related gene Atg5 after uric acid exposure. B. LC3I, LC3II, and p62 protein levels in H9c2 cardiomyocytes with different concentrations of uric acid treatment were evaluated by western blotting. C. Densitometric analysis showed that uric acid treatments gradually increased the ratios of LC3II/LC3I and decreased the p62 expression in a dose-dependent manner. The data are expressed as mean  $\pm$  SD; \*P < 0.05 and \*\*P < 0.01 indicate significant differences compared to the control group.

changes. Myocardial hypertrophy is an initial adaptive process of cardiomyocytes in response to various internal and external changes. With prolonged and abnormal stimulation, this compensatory response eventually becomes irreversible [20, 24]. Maladaptive myocardial hypertrophy leads to a decrease in cardiac function and the development of heart failure [25]. Thus, it is reasonable to hypothesize that longterm hyperuricemia is accompanied by a decrease in left ventricular function.

Indeed, a high serum uric acid level (serum uric acid > 8 mg/dl) is independently associated with heart failure and left ventricular enlargement in individuals with hyperuricemia accompanied by early onset cardiovascular diseases [6]. Subjects with a mean disease duration of 2.3 ± 7.8 years of asymptomatic hyperuricemia showed a deterioration in the parameters that reflected left ventricular function, such as stroke volume, global longitudinal strain, and global circumferential strain as reflected by three-dimensional speckle tracking echocardiography [26]. More importantly, global circumferential strain, which is a marker that increases with declining left ventricular function, was positively associated with the level of serum uric acid in subjects with hyperuricemia either with or without hypertension [14, 26]. We found that the hypertrophic effect of uric acid was dose-dependent, and that, the effect was enhanced with increasing uric acid



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**Figure 3.** Soluble uric acid induces hypertrophy in H9c2 cardiomyocytes through activation of autophagy. H9c2 cardiomyocytes were treated with uric acid in the absence or presence of 5 mM 3-MA (pretreated for 1 hour). A. Real-time PCR analysis of the mRNA expression of the hypertrophy related genes *Nppa*, *Nppb*, and *Myh7*. B. Cells were hematoxylin-eosin stained and visualized with a microscope. Bars indicate for 20  $\mu$ m. C. The cell surface areas were measured using ImageJ software. The data are expressed as mean ± SD; \*P < 0.05 and \*\*P < 0.01 indicate significant differences compared to the control group. UA, uric acid; 3-MA, 3-methyladenine.



Figure 4. Soluble uric acid activates AMPK-ULK1 in H9c2 cardiomyocytes. H9c2 cardiomyocytes were treated with uric acid. A. Western blot analysis of the protein expression of phosphorylated of AMPK and ULK1 at different time point of uric acid treatment. B. Densitometric analysis showed that uric acid increased the ratios of p-AMPK/AMPK and p-ULK1/ULK1. The data are expressed as mean  $\pm$  SD; \*P < 0.05 and \*\*P < 0.01 indicate significant differences compared to the control group.

concentrations. Thus, it is reasonable to hypothesize that the decline in left ventricular function observed in subjects with hyperuricemia was mediated by uric acid-induced myocardial hypertrophy.

Our results show that uric acid-induced myocardial hypertrophy is mediated by autophagy. Cardiac autophagy is regulated by various factors such as calorie restriction, pressure overload, and berberine treatment [27-29]. Excessive cardiac autophagy leads to cardiac hypertrophy and heart failure [30]. Our findings uncover a new factor, uric acid, that stimulates autophagy in cardiomyocytes and that activation of autophagy is responsible for myocardial hypertrophy. The present study revealed a pathogenic role of uric acid in cardiomyocytes through elevated autophagy. Autophagy was measured after 48 hours of stimulation by uric acid. However, insufficient autophagy has been observed after approximately 2 to 4 weeks in a mouse model of pressure overload [31]. Thus, it is reasonable to hypothesize that longterm stimulation with a high concentration of uric acid may weaken the activation of auto-



**Figure 5.** AMPK is involved in the activiation of autophagy induced by uric acid. H9c2 cardiomyocytes were treated with uric acid in the absence or presence of 5  $\mu$ M Compound C (pretreated for 1 hour). A. Real-time PCR analysis of the mRNA expression of the autophagy related gene *Atg5*. B. LC3I, LC3II, and p62 protein levels in H9c2 cardiomyocytes were evaluated by wetern blotting. C. Densitometric analysis showed that Compound C significantly attenuated the increased ratios of LC3II/LC3I and decreased p62 expression induced by uric acid. D. Real-time PCR analysis of the mRNA expression of the hypertrophy related genes *Nppa*, *Nppb*, and *Myh7*. E. Cells were hematoxylin-eosin stained and visualized with a microscope. Bars indicate for 20  $\mu$ m. F. The cell surface areas were measured using ImageJ software. The data are expressed as mean  $\pm$  SD; \*P < 0.05 and \*\*P < 0.01 indicate significant differences compared to the control group. UA, uric acid; CC, Compound C.

phagy, thereby leading to cardiac damage. Further *in vivo* studies are needed to test this hypothesis.

In this study, we found that uric acid phosphorylated and activated AMPK. The activity of AMPK is either activated or inhibited by uric acid, depending on the cell type [15-17]. The phosphorylation and activation of AMPK at Thr172 by uric acid has been reported in pancreatic  $\beta$  cells and PMA-primed THP-1 cells [17]. We found that uric acid also increased the activity of AMPK in H9c2 cardiomyocytes. As an important pro-oxidant within cells, increased levels of reactive oxygen species upon uric acid treatment have been reported to be responsible, at least in part, for the activation of AMPK [15, 16]. However, in our study, reactive oxygen species were not measured. But we observed a reduced level of ATP (data not shown), which is an activator of AMPK, after treatment with uric acid. Thus, in cardiomyocytes, depletion of ATP may underlie the uric acid-induced activation of AMPK.

After further analysis, we found that the increased AMPK activity in cardiomyocytes was responsible for the activation of autophagy, as the inhibition of AMPK by Compound C completely abolished the increased autophagy and



**Figure 6.** Schematic diagram of the signaling pathway involved in uric acid-induced myocardial hypertrophy. Uric acid induces phosphorylation and activation of AMPK, which further phosphorylates ULK1. Activation of ULK1 increases autophagy activity, which ultimately contributes to myocardial hypertrophy.

subsequent myocardial hypertrophy. AMPK-mediated activation of autophagy occurred either through the AMPK-mTORC1-ULK1 pathway or the AMPK-ULK1 pathway [32]. We found increased phosphorylation of ULK1 at Ser555, a site that is directly phosphorylated by AMPK [33], upon uric acid stimulation. Thus, in cardiomyocytes, AMPK directly phosphorylated ULK1, which further enhanced the autophagy upon uric acid stimulation.

It should be noted that it remains unclear whether uric acid is directly transported into cardiomyocytes to exert its effects. We examined the mRNA expression of some of the uric acid transporters in H9c2 cardiomyocytes and detected the expression of *ABCG2*, the product of which is responsible for the excretion of uric acid from tubular cells to lumens [34]. The expression of *Urat1* and *Glut9*, whose products are responsible for urate reabsorption [35, 36], was undetectable (data not shown). Further indepth studies are needed to determine whether cardiomyocytes directly take up uric acid.

There were several limitations in the current study. First, H9c2 cardiomyocytes are a rat myoblast-derived cell line, and the response may differ from that of primary cardiomyocytes upon stimulation. Thus, studies using primary cardiomyocytes are needed to confirm the current findings. Second, phosphorylation of raptor was not investigated. AMPK phosphorylates raptor, which inhibits the activity of mTORC1, a negative regulator of ULK1 [37]. Thus, in addition to direct activation by AMPK, it is not known whether the increased activity of ULK1 was partly due to the inhibition of mTORC1. Third, the levels of reactive oxygen species were not measured. Therefore, whether the stimulation of AMPK was due to increased levels of reactive oxygen species were not determined. Finally, autophagosomes were not measured by electron microscopy in our study. However, with the real-time PCR and western blotting findings, we did find increased autophagy activity upon uric acid treatment.

In conclusion, uric acid induced myocardial hypertrophy through the activation of autophagy. The increased activity of the AMPK-ULK1 signaling pathway was responsible for uric acid-induced autophagy and subsequent myocardial hypertrophy (**Figure 6**). Our findings reveal a new mechanism by which a high concentration of uric acid influences cardiomyocytes. Decreasing the serum uric acid level may therefore be clinically beneficial for alleviating cardiac hypertrophy. However, additional in-depth studies are needed to uncover the relationship between uric acid-induced myocardial hypertrophy and cardiac function.

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# Disclosure of conflict of interest

#### None.

Address correspondence to: Lin-Di Jiang, Department of Rheumatology, Zhongshan Hospital,

Fudan University, Shanghai 200030, China. Tel: +86-021-31582621; Fax: +86-021-31582621; E-mail: zsh-rheum@hotmail.com

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