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Original Article

Uric acid promotes cardiomyocyte apoptosis through regulating microRNA-21/PTEN/AKT/eNOS axis in myocardial ischemia/reperfusion injury

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Abstract: Myocardial ischemic/reperfusion injury (MI/RI) results from severe impairment of coronary blood supply and leads to irreversible cell death, with limited therapeutic possibilities. Uric acid (UA) is a purine nucleotide metabolite that is synthesized in the liver from xanthine via xanthine oxidase and affects various vasoactive mediators. The aim of this study was to investigate the effect of UA on cardiomyocyte apoptosis and its molecular mechanism in MI/RI. H9c2 cells were treated with UA after which cell viability, apoptosis, and caspase activation were evaluated by CCK-8, flow cytometry and Western Blotting. Meanwhile, eNOS expression and NO content were measured using the fluorescent indicator DAF-FM diacetate and Cayman's NOS activity assay kit. Then, the roles of UA on miR-21 expression were investigated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Further, the potential target genes of miR-21 were predicted by bioinformatics analyses and confirmed by dual-luciferase reporter assay. Finally, H9c2 cells were transfected with miR-21 inhibitor or mimic to confirm the role of miR-21 on the protein expression of PTEN/AKT/eNOS pathway and UA-induced apoptosis. Our study showed that UA inhibited H9c2 cells growth in a concentration- and time-dependent manner. UA at 15 mg/dl effectively promoted apoptotic cell death, increased the activities of caspase-3 and PARP, which was along with dysregulation of Bcl-2 and Bax protein in activated apoptotic pathway. In addition, UA caused a dose-dependent decrease in the NO production through inhibiting eNOS phosphorylation in cultured H9c2 stimulated by Bradykinin. We further demonstrated that UA inhibited miR-21 expression in a concentration- and time-dependent manner and PTEN was identified as a target of miR-21. Knockdown of miR-21 decreased eNOS and AKT phosphorylation and miR-21 overexpression could alleviate the cell apoptosis induced by UA in H9c2 cells. Collectively, the findings that UA inhibits cardiomyocyte growth, call for careful reconsideration of the role of UA in MI/RI. Our data also show that UA increases cardiomyocyte apoptosis through regulating miR-21/PTEN/AKT/eNOS axis, indicating that interruption of miR-21/PI3K/Akt/eNOS may represent a novel therapeutic strategy in MI/RI.

Keywords: Uric acid, myocardial ischemia/reperfusion injury, microRNA-21, apoptosis, PTEN, PI3K/AKT/eNOS signaling pathway

Introduction

As the changes of lifestyle and the ageing of population, acute myocardial infarction has been one of the major reasons for the threat to the life and health of human [1, 2]. Although essential for successful interventional therapy, it is inevitably complicated by reperfusion injury. During ischemia, it was reported that myocardial ischemia/reperfusion injury (MI/RI) was related to the release of oxygen free radical, overload of intracellular calcium, metabolic disorders of myocardial energy, invasion of neutrophils, injury of vascular endothelial cell and apoptosis [3-6]. Of these, apoptosis is one of the most significant mechanisms of I/R injury, as a recent study demonstrated that MI/RI occurs in proportion to apoptosis [7]. Thus effective approaches to reduce or eliminate ischemia/reperfusion injury have become a primary focus of both basic and clinical research.

Uric acid (UA) is the final product of purine nucleotide metabolite. Elevated serum UA levels have been strongly associated with cardiovascular diseases including ischemic heart disease, heart failure and stroke [8-10]. However, the role of uric acid in MI/RI remains largely

unknown. Recently, a study from Duk-Hee Kang et al. shows that UA inhibits nitric oxide (NO) production and alters the expression of C-reactive protein (CRP), which has emerged as one of the most powerful independent predictors of cardiovascular disease, leading to endothelial cell apoptosis [11]. Papežíková I et al. also showed that UA dose-dependently inhibits NO production in cultured aortic endothelial cells [12]. Since UA decreased NO production, we hypothesize that UA may impair NO production in cardiomyocytes and in this way contributes to the severity of MI/RI.

MicroRNA (miRNA) is a class of endogenous non-coding single and small RNA which is composed with 21-23 bases that negatively regulating the gene expression at post-transcriptional level by degrading target mRNA or translation repression [13]. Studies found that the expression of miRNA was rich in the cardiovascular system, involved in heart development, myocardial cell apoptosis, arrhythmia, heart failure and other pathological and physiological processes. And, kinds of miRNAs play an important role in ischemia/reperfusion injury [14-16]. MiR-21 was shown to prevent myocardial apoptosis against ischemia/reperfusion (I/R)-induced cardiomyocyte injury [17] and reduced cell apoptosis and myocardial infarct size at an early stage of acute myocardial infarction (AMI) [18]. However, whether miR-21 participates in the pro-apoptotic effects of UA is unknown.

In the current study, we investigated the effects of UA on apoptosis and the underlying mechanisms in cardiomyocyte. Our results show that UA promotes cardiomyocytes apoptosis by reducing NO production. Furthermore, we demonstrate that miR-21 contributes to the pro-apoptotic effects of UA in cardiomyocyte via PI3K/AKT/eNOS signaling pathway by targeting PTEN. The results of the present study may help to provide a new therapeutic target for the treatment of MI/RI.

Materials and methods

Reagents and antibodies

Uric acid was purchased from Sigma-Aldrich (Carlsbad, CA). Antibodies against cleaved caspase-3, cleaved-PARP, Bcl-2, Bax, Akt and phospho-Akt (p-AKT-Ser^{473}) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against eNOS and p-eNOS-Ser^{1177} were purchased from Transduction Laboratories (Lexington, KY). Antibody against PTEN was purchased from Cell Signaling Technology, Inc., (Cell Signaling, Danvers, MA, USA). Anti-β-actin antibody was obtained from Sigma (St. Louis, MO).

Cell culture

H9c2 cells were purchased from American Type Culture Collection (ATCC, CRL-1446) (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Shanghai, China) containing 10% fetal bovine serum (FBS, Life Technologies), 1% streptomycin (100 μg/mL) and 1% penicillin (100 U/mL) at 37°C under an atmosphere of 5% CO_2.

Cell viability assay

To assess cellular viability, 1×10^3 H9c2 cells were added to the wells of a 96-well plate. The different groups were pretreated with various concentration of UA (5, 10, 15 mg/dl). Viability of cardiomyocyte was detected using the CCK-8 assay at different time (12, 24, 48 hrs). Briefly, in the absence of light, CCK-8 reagent (10 μl) was added to each well and the plates were incubated at 37°C under an atmosphere of 5% CO_2 for 1-4 h. Then, the optical densities were detected using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Apoptosis assay

H9c2 cells were plated in a 6-well plate at a concentration of 3×10^5 cells per well. After various concentration of UA (5, 10, 15 mg/dl) treatment, the cells were washed with 1×PBS for three times. Then, an Annexin-V FITC-PI Apoptosis Kit (Invitrogen) was applied to determine the apoptotic rate by flow cytometry. This assay employs fluorescein-labeled Annexin-V in concert with propidium iodide (PI) to detect the cells undergoing apoptosis.

Measurement of NO production

NO production was determined by using the NO-specific fluorescent dye 4, 5-diaminofluorescein diacetate (DAF-2 DA; Cayman Chemical, Ann Arbor, MI, USA) [19]. H9c2 cells were incubated in 96-well black-wall, clear-bottom plates. After treatment, cells were loaded with DAF-2 DA (1 μM) for 30 min at 37°C and rinsed

3 times with Dulbecco's modified Eagle's medium. The cells were fixed in 4% paraformaldehyde for 5 min. The fluorescence was measured with a microplate fluorescence reader (Molecular Devices, Sunnyvale, CA, USA) at excitation 480 nm and emission 510 nm.

Determination of eNOS activity in the total membrane fraction from H9c2

eNOS activity was measured by Cayman's NOS activity assay kit (Ann Arbor, MI), according to the manufacturer's instructions, as described previously [20]. Control H9c2 and H9c2 treated with UA were scraped and homogenized in buffer A (50 mM Tris·HCl, pH 7.4, containing 0.1 mM EDTA and EGTA, 1 mM PMSF, and 1 μg/ml leupeptin). The homogenates were centrifuged at 100,000 g for 60 min at 4°C, and the total membrane fraction pellet was resuspended in buffer B (buffer A+2.5 mM $CaCl_2$). eNOS activity in the total membrane fraction was determined by monitoring the formation of L-[^{3}H]citrulline from L-[^{3}H]arginine in buffer A with the addition of UA, 1 mM NADPH, 100 nM calmodulin, 10 μM BH4, and 5 μM L-arginine containing purified L-[^{3}H]arginine for 30 min at 37°C. The measurement of L-[^{3}H]citrulline formation was performed as previously described [19].

RNA extraction and quantitative reverse transcription PCR

Total RNA was isolated using TRIzol (Invitrogen, CA) and miRNeasy mini kit (Qiagen, West Sussex, UK) according to manufacturer's instructions. Total RNA from each sample was reverse-transcribed to cDNA using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan). RT-qPCR was performed using an ABI7500 real-time DNA detection system (Applied Biosystems, Waltham, MA, USA) with SYBR Green Master Mix (TIANGEN Biotech (Beijing) Co., Ltd., Beijing, China). The primers for miR-21 and U6 were designed by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). The sequences of primers were: miR-21 forward: 5'-GGGGTAGCTTATCAGATCG-3' and reverse: 5'-TGGAGTCGGCAATTGCACTG-3'; U6 forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'. All samples were analyzed in triplicate. The relative expression levels of miR-21 were detected by the standard curve method, and normalized against U6.

Transfection

The cells were plated into six-well plates and grown to 30-50% confluence after 24 hours of incubation and were then transfected with a specific miR-21 mimics, a miR-21 inhibitor or negative control at a final multiplicity of infection of 10 using siLentFect™ Lipid reagent (Life Science Research). The cells were then diluted in DMEM/F12 without serum (GeneChem, Shanghai, China). After 4 h of incubation in a CO_2 incubator at 37°C, the medium was changed to 10% FBS containing DMEM.

Luciferase reporter assay

Dual luciferase assays were conducted in a 24 well plate format. pGL3-PTEN 3'UTR report/pGL3-PTEN 3'UTR Mutant report +TK100 Renilla report were transfected into 70% confluent H9c2 cells, along with miR-21 mimic, miR-21 inhibitor or each control. After 48-h transfection, firefly and renilla luciferase were quantified sequentially using the Dual Luciferase Assay kit (Promega, USA) following the manufacturer's recommendations.

Western blot analysis

Protein extracts from H9c2 cells were subjected to 10% SDS-PAGE and subsequently transferred to a PVDF membrane. The membrane was blocked with 5% (w/v) nonfat milk and incubated sequentially with the primary antibodies in TBST containing 5% bovine serum albumin overnight at 4°C. Anti-β-actin antibody was used as an internal control. After washing three times with TBST, the membrane was incubated at room temperature for 2 hours with horseradish peroxidase-conjugated secondary antibody (anti-rabbit, 1:2000, Cell Signaling Technology) diluted with TBST. The detected protein signals were visualized using an enhanced chemiluminescence (ECL) system western blot kit (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Statistical analyses were performed with SPSS 13.0 software. The results were evaluated by χ^2 test and the other data were evaluated by Student's t-test and expressed as the mean ± SD from three independent experiments. A *P*-value of less than 0.05 was considered statistically significant.

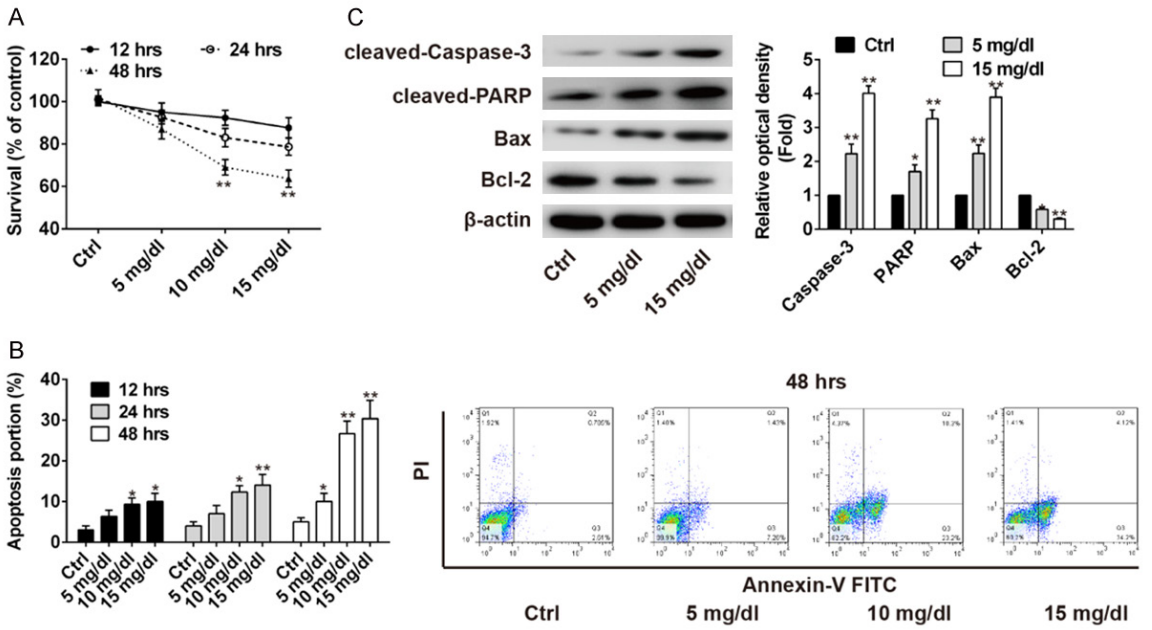

Figure 1. UA inhibited cell growth and promoted cell apoptosis in H9c2 cells. A. Effects of increasing doses of UA on H9c2 viability at 12, 24 and 48 hours. A decrease in cell viability was observed with higher concentrations (5-15 mg/dl). B. Effects of increasing doses of UA on H9c2 apoptosis at 12, 24 and 48 hours. An increase in cell apoptosis was observed with higher concentrations (5-15 mg/dl). C. The levels of apoptosis-relative proteins including cleaved caspases 3, cleaved PARP, Bcl-2 and Bax are assessed by Western Blot after treatment with increasing doses of UA. Data are presented as mean ± SD from three independent experiments. *P < 0.05, **P < 0.01 vs. control.

Results

UA suppresses the cell growth and promotes apoptosis in H9c2 cells

To investigate the effect of UA on the growth of cardiomyocytes, H9c2 cells were treated with UA at different concentration (5 to 15 mg/dl) for 12, 24 and 48 h, and then the cells were analyzed by CCK-8 Assay. As shown in the **Figure 1A**, the growth of H9c2 cells was inhibited in a dose- and time-dependent manner, and the maximal inhibitory effects were afforded by 15 mg/dl UA for 48 h.

To explore whether UA-induced cytotoxicity also involves apoptosis, we have assessed the effect of UA on H9c2 cell apoptosis with flow cytometry. As shown in **Figure 1B**, compared with the control group, apoptosis was markedly increased in H9c2 cells in a dose- and time-dependent manner. Similar with the above result, the maximal promotional effects were also afforded by 15 mg/dl UA for 48 h. Therefore, we used 15 mg/dl UA and 48 h to further investigate its pro-apoptotic effects in H9c2 cardiomyocytes.

It is well known that the inhibition of caspase functions has been shown to block the development of cell apoptosis. Therefore, we tested whether the apoptosis of H9c2 cells caused by UA was associated with the activation of caspase-3 and PARP. To address this question, Western blot were used to examine the expression changes of cleaved-caspase-3 and cleaved-PARP. And, we found that cleaved-caspase-3 and cleaved-PARP was markedly increased in the cells incubated with UA, which was along with dysregulation of Bcl-2 and Bax protein in activated apoptotic pathway (**Figure 1C**). These results suggest that the UA could induce apoptosis in cardiomyocytes by caspase-dependent process.

UA reduces NO production through modulating eNOS activity

It has reported that UA modulates vascular endothelial function through the down regulation of NO production [11]. Thus, we assumed that UA induces the apoptosis of cardiomyocytes via regulation of NO production. We tested the effects of UA on production of NO by cultured H9c2 cells using the fluorescent probe

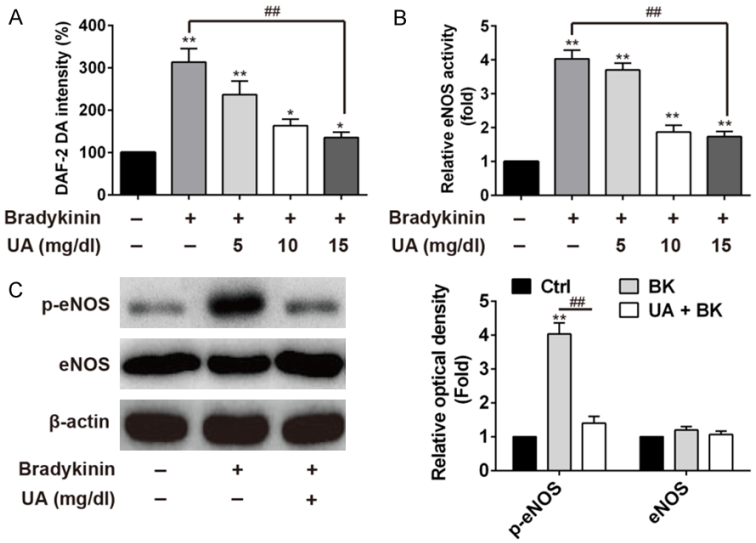

Figure 2. UA decreases NO release and eNOS activity in H9c2 cells in a dose-dependent manner. Confluent monolayers of H9c2 cells were treated with UA at various doses (5, 10, and 15 mg/dl) for 24 h. Subsequently, bradykinin (BK, the final concentration of 2 μM) was added to cells to stimulate NO production. A. NO detection using DAF-FM was performed as described in Materials and methods. B. eNOS activity in the cells treated without or with uric acid for 24 h was measured by Cayman's NOS activity assay kit as described in Materials and methods. C. Western blot analysis of eNOS and p-eNOS was performed in H9c2 cells. Data are presented as mean ± SD from three independent experiments. **P < 0.01 vs. control; ##P < 0.01 vs. BK group.

DAF-FM [21], which can detect real-time changes in intracellular NO following stimulation of NO production with agents such as ACh, bradykinin, or calcium ionophores [19]. As expected, UA causes a dose-dependent decrease in the NO production in cultured H9c2 stimulated by 2 μM bradykinin (BK) (**Figure 2A**). Subsequently, we found that UA clearly decreased eNOS activity (**Figure 2B**), indicating that decreased NO production is due to decreased eNOS activity.

To further explore the mechanism by which UA decreases eNOS activity, we examined whether UA decreases eNOS expression. Cultured H9c2 cells were incubated for 24 h with different concentrations of UA. After incubation, Western blot analysis was assayed. Interestingly, UA decreased the BK-induced phosphorylation of eNOS at Ser^{1177} (**Figure 2C**). However, UA did not alter the levels of eNOS. These data demonstrate that UA affect eNOS activity through inhibiting the phosphorylation of eNOS.

UA decreases the expression of miR-21 in H9c2 cells

Recent studies have provided direct evidence of microRNA-21 involvement in cell apoptosis modulation in different type of cells [22, 23]. Previous study showed that the production of NO by eNOS was regulated by microRNA-21 [20]. Based on the above results, we hypothesized that miR-21 was involved in cardiomyocyte apoptosis induced by UA. To test this hypothesis, H9c2 cells were treated with UA at different concentration (5 to 15 mg/dl) for 12, 24 and 48 h, then, the level of miR-21 was determined by qRT-PCR. As shown in **Figure 3A** and **3B**, UA decreased the level of miR-21 in a dose- and time-dependent manner.

It has reported that PTEN was a direct target of miR-21 in many diseases and cell types [22, 24-26]. However, whether PTEN is a direct target of miR-21 in cardiomyocyte is still unknown. Therefore, we cloned a sequence with the predicted target sites of miR-21 or a mutated sequence with the predicted target sites to downstream of the pGL3 luciferase reporter gene and employed Western blotting assay. When wild-type (wt) or mutant vector was transfected with miR-21 inhibitor, the luciferase activity of wt vector was significantly increased compared with mutant vector, whereas the luciferase activity of wt vector was significantly decreased when transfected with miR-21 mimic (**Figure 3C**). By contrast, there was no significant difference between wt and mutant vector when transfected with negative control miRNA. In addition, we found that transfection with the miR-21 mimic decreased PTEN protein levels, while transfection with the miR-21 inhibitor increased PTEN protein levels (**Figure 3D**). These results indicated that PTEN was a direct target of miR-21 in cardiomyocyte.

Inhibition of miR-21 suppressed the activity of PI3K/AKT/eNOS signaling pathway

It is reported that PTEN gene inhibits Akt activation (phosphorylation) [27, 28], and the PI3K/Akt pathway is involved in decreasing apoptosis in various cells types. As is reported, one of the major targets of Akt is eNOS, which cata-

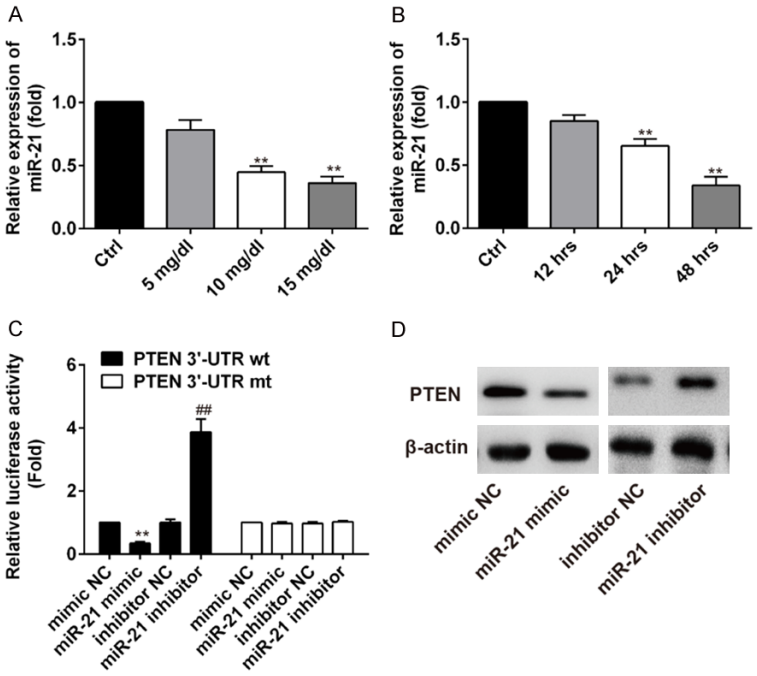

Figure 3. UA inhibits miR-21 expression in H9c2 cells. A and B. qRT-PCR detected miR-21 expression in H9c2 cells treated with different concentrations UA (5-15 mg/dl) at 12, 24 and 48 hours. UA inhibits miR-21 expression in a dose- and time-dependent manner. Data are presented as mean ± SD from three independent experiments. **P < 0.01 vs. control. C. Luciferase activities. H9c2 cells were co-transfected with firefly luciferase constructs containing the PTEN wild-type or mutated 3'-UTRs and miR-21 mimic, mimic NC, miR-21 inhibitor or inhibitor NC, as indicated (n = 3). D. The protein expression of PTEN after treatment with miR-21 mimic or miR-21 inhibitor (n = 3). Data are presented as mean ± SD from three independent experiments. ##P < 0.01 vs. mimic NC group; **P < 0.01 vs. inhibitor NC group.

lyzes L-arginine to produce nitric oxide (NO) and is associated with the cardio-protection in myocardium I/R injury [29]. As shown in **Figure 4A** and **4B**, we found that expression of phosphorylation of Akt (Ser^{473}), and eNOS (Ser^{1177}) were decreased significantly after transfection with miR-21 inhibitor. This data suggested that knockdown of miR-21 inhibited activity of the PI3K/Akt/eNOS pathway in cardiomyocytes through upregulation of PTEN, a negative regulator of the PI3K/Akt/eNOS pathway.

UA induced cardiomyocytes apoptosis by mediating miR-21 expression

To examine whether the expression of miR-21 contributes to the UA-induced apoptosis in cardiomyocytes, we transfected miR-21 mimic to H9c2 cells, and then observed the alteration of UA-induced growth and apoptosis in cardiomyocytes. As shown in **Figure 5A**, miR-21 mimic restored the reduction of cell viability induced by UA. Moreover, miR-21 mimic alleviated the promotion of cell apoptosis induced by UA (**Figure 5B**), indicating that the elevation of miR-21 is involved in the process of UA-induced apoptosis in cardiomyocytes.

Discussion

In the present study, we demonstrated that UA could inhibit the growth and induced apoptosis of cardiomyocytes in a dose- and time-dependent manner. And we found that UA altered NO release of cardiomyocytes via modulating the activity of eNOS. Furthermore, we show that UA induced cardiomyocyte apoptosis is mediated by the miR-21/PI3K/Akt/eNOS pathway in MI/RI. Our study implies that miR-21/PI3K/Akt/eNOS pathway may be a new therapeutic candidate in the treatment of MI/RI.

Although improvements in surgical care, it is inevitably complicated by reperfusion injury in the process of myocardial I/R [30, 31]. Several reports have demonstrated that high concentration of UA could increase apoptotic activity in different cells [32, 33]. For example, Daniela Verzola et al. found that UA increased the permissiveness of proximal tubule kidney cells to apoptosis by triggering a pathway involving NADPH oxidase signaling and URAT 1 transport [33]. It has been reported that myocardial apoptosis may attribute to cell death following acute myocardial infarction/reperfusion. Recent study showed that UA dose-dependently decreased NO production in intact bovine aortic endothelial cells, which was a hallmark of endothelial dysfunction [34]. In this study, we tested the hypothesis that UA induced changes in cardiomyocytes apoptosis mediated by production of NO. Our results suggested that cardiomyocytes treated with high concentrations of UA for extended periods displayed a decrease in cell growth and an increase in

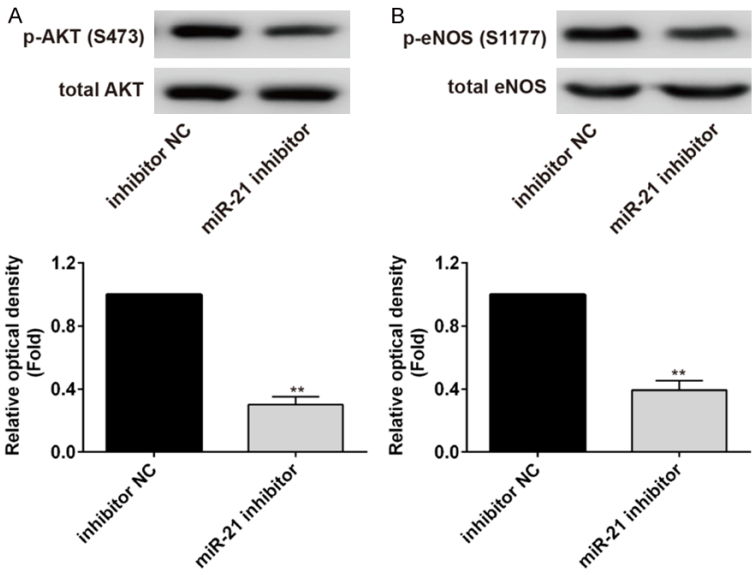

Figure 4. The impact of miR-21 knockdown in H9c2 cells on phosphorylation of AKT and eNOS. A. Western analysis and densitometry of p-AKT (serine 473) after transfection with miR-21 inhibitor for 48 h. B. Western and densitometry of p-eNOS (serine 1177) after transfection with miR-21 inhibitor for 48 h. Data are presented as mean ± SD from three independent experiments. **P < 0.01 vs. inhibitor NC group.

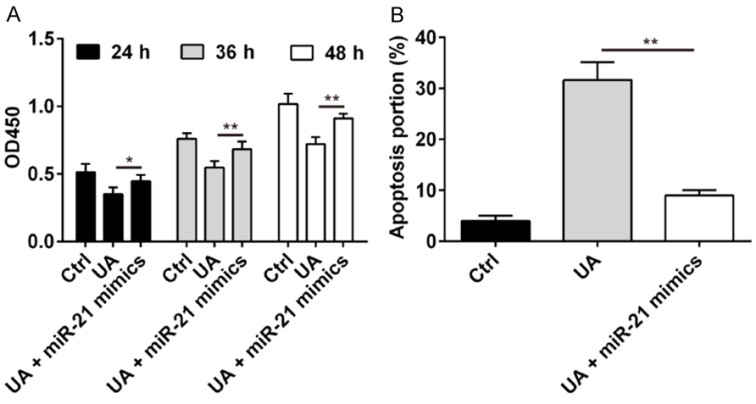

Figure 5. Overexpression of miR-21 alleviates the pro-apoptotic effects of UA in H9c2 cells. A. Overexpression of miR-21 restores the reduction of cell viability induced by UA in H9c2 cells. n = 3. B. Overexpression of miR-21 alleviates the promotion of cell apoptosis induced by UA in H9c2 cells. Data are presented as mean ± SD from three independent experiments. **P < 0.01 vs. mimic NC group.

apoptosis. Furthermore, we demonstrated that UA decreased NO production by inhibiting the activity of eNOS. These results revealed that UA has pro-apoptotic potency in cardiomyocytes.

In recent years, the function of miRNAs in MIRI was paid to more attention. Ren XP et al. found that the expression of miRNA-320 was significantly decreased in MIRI and knockout of miRNA-320 increased the expression of HSP20, reducing myocardial cell apoptosis induced by myocardial ischemia/reperfusion [35]. A study performed by Xu C et al. showed that miR-1 and miR-133 produced opposing effects on oxidative stress-induced apoptosis in H9c2 cells, with miR-1 being pro-apoptotic and miR-133 being anti-apoptotic [36]. Increasing evidence indicates the importance of miRNAs in the regulation of cardiac developmental and pathological processes [37-40]. In this present study, we found that UA inhibited the expression of miR-21, an abundant miRNA in cardiomyocytes in a dose- and time-dependent manner. More importantly, PTEN was identified as a target of miR-21, a negative regulator of the PI3K/Akt/eNOS pathway [41]. However, it is unknown whether miR-21 mediated the pro-apoptotic effect of UA.

Previous studies demonstrated that some signaling molecules were thought to serve as the upstream mediators of apoptosis during MIRI including the PI3K/Akt pathway [42, 43]. Several recent studies have demonstrated that insulin and other hormones such as estrogen cause eNOS phosphorylation and result in endothelial NO release through the PI3K/Akt dependent pathway [44]. In the present study, the phosphorylation levels of Akt and eNOS were greatly inhibited by miR-21 inhibitor, indicating that miR-21 could modulate the PI3K/Akt/eNOS pathway in cardiomyocytes. In addition, we also found that overexpression of miR-21 could restore the reduction of cell viability and alleviated the promotion of cell apoptosis induced by UA. These data sug-

gest that the downregulation of miR-21 is involved in the process of UA-induced apoptosis in cardiomyocytes.

In summary, our data, UA alters growth/apoptosis and NO release of cardiomyocyte, indicating that UA may promote the development and progression of MI/RI. Furthermore, UA induced cardiomyocyte apoptosis is mediated by the miR-21/PI3K/Akt/eNOS pathway, suggesting that miR-21/PI3K/Akt/eNOS pathway may be a new therapeutic candidate in the treatment of MI/RI.

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Disclosure of conflict of interest

None.

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