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Gracillin inhibits apoptosis and inflammation induced by lipopolysaccharide (LPS) to alleviate cardiac injury in mice via improving miR-29a

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ABSTRACT

Sepsis induces critical myocardial dysfunction, resulting in an increased mortality. Gracillin (GRA) is a natural steroidal saponin, showing strong capacities of anti-inflammation, but its pharmacological effects on lipopolysaccharide (LPS)-induced acute cardiac injury still remain unclear. In this study, we attempted to explore if GRA was effective to attenuate cardiac injury in LPS-challenged mice and the underlying mechanisms. First, we found that GRA treatments markedly up-regulated the expression of miR-29a in cardiomyocytes. LPS-induced cytotoxicity in cardiomyocytes was significantly alleviated by GRA treatment, as evidenced by the improved cell viability and reduced lactate dehydrogenase (LDH) release. In addition, LPS-triggered apoptotic cell death was clearly ameliorated in cardiomyocytes co-treated with GRA. Notably, LPS-exposed cells showed significantly reduced expression of miR-29a, while being rescued by GRA treatment. *In vivo*, LPS apparently impaired cardiac function in mice, which was, however, alleviated by GRA administration. In addition, GRA markedly attenuated apoptosis in hearts of LPS-challenged mice by decreasing the expression of cleaved Caspase-3. LPS-triggered inflammatory response in cardiac tissues was also suppressed by GRA through blocking nuclear factor κB (NF-κB) signaling pathway. We also found that miR-29a expression was highly reduced in hearts of LPS-treated mice but was rescued by GRA pretreatment. Besides, miR-29a mimic alleviated LPS-induced apoptosis and inflammation in cardiomyocytes; however, LPS-caused effects were further accelerated by miR-29a. Of note, the protective effects of GRA on LPS-injured cardiac tissues were significantly abrogated by miR-29a suppression. In conclusion, our findings demonstrated that GRA exerted an effective role against LPS-induced acute cardiac injury through impeding apoptosis and inflammation regulated by miR-29a.

1. Introduction

Sepsis is known as a systemic disease caused by an inappropriate immune response. Myocardial dysfunction is a common complication of severe sepsis, resulting in frequent death in intensive care units [1]. Numerous mechanisms have been proposed to be involved in septic cardiomyopathy, such as the uncontrolled immune and inflammation, mitochondrial energy metabolic disorders, oxidative stress and apoptosis [2,3]. Presently, some therapeutic strategies have been used to prevent sepsis-induced cardiac injury, including heart transplantation, angiotensin inhibitors and diuretics [4]; however, the specific interventions that target sepsis-triggered cardiac dysfunction and heart injury are still lacking.

MicroRNAs (miRNAs) are reported as a class of short noncoding 10 to 25 nucleotide long RNA, and have been implicated in the modulation of physiological and pathological processes [5]. Accumulating studies have showed that miRNAs are responsible for the improvement of cardiac dysfunction associated with sepsis [6,7]. Aberrant expression of miRNA has been proposed as predictive biomarkers and prognostic factors that are associated with the progression of cardiovascular disease, including myocarditis caused by LPS [8]. For instance, in LPS-stimulated cardiomyocytes, miR-203

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could enhance apoptosis and inflammation [9]. In addition, miR-93−3p attenuated LPS-induced inflammation and cell death in cardiomyocytes through suppressing toll-like receptor 4 (TLR4) signaling [10]. MiR-29 family, consisted of three mature members including miR-29a, miR-29 b, and miR-29c, is encoded by two genome clusters. Specific overexpression of miR-29 in fibroblasts decreased collagen expression during the left ventricular (LV) repairing process [11]. Additionally, in myocardial-infarcted rodent animals, the controlled intermittent aerobic exercise could reduce fibrosis through increasing the expression of miR-29a to ameliorate heart injury [12]. However, if miR-29a participates in sepsis-associated cardiac injury and the possible mechanism still remain unclear.

Various kinds of natural products have been identified as important sources for the development of agents against sepsis-induced heart injury. Gracillin (GRA, Fig. 1A) is a natural steroidal saponin component of *Dioscorea quinqueloba* (*D. quinqueloba*) [13]. Saponin has been reported to possess anti-tumor, anti-microbial, anti-oxidant, anti-apoptosis and anti-inflammation properties [14,15]. Recently, GRA was suggested to show anti-inflammatory effects against skin disorders *in vitro* and *in vivo* [16]. More recently, GRA exhibited anti-cancer property partly through regulating apoptosis [17]. These findings have implied the promising benefits of GRA in the treatment of sepsis-induced myocardial dysfunction. However, no experiments have been performed to investigate the effects of GRA on LPS-induced heart damage. Therefore, we attempted to address this question and reveal the underlying molecular mechanisms. In the present study, we found that LPS reduced miR-29a expression, contributing to the progression of apoptosis and inflammation. However, these effects were reversed by GRA treatment, attenuating cardiac dysfunction and acute heart injury.

2. Materials and methods

2.1. Materials and reagents

Lipopolysaccharide (LPS) was obtained from Sigma (Cat. No. L3012; St Louis, MO, USA). Gracillin (GRA, Fig. 1A) for *in vitro* and *in vivo* treatments was purchased from Selleck (purity > 98%; Cat. No. S3915; USA). The miR-Con (5′-UUCUCCGAACGUGUCACGUTT-3′), miR-29a mimic (5′-UAGCACCAUCUGAAAUCGGUUA-3′), miR-29a scramble (5′-CAGUACUUUUGUGUAGUACAA-3′) and miR-29a inhibitor (5′-UAACCGAUUUCAGAUGGUGCUA-3′) were synthesized and acquired from RiboBio (Guangzhou, China). TRIzol® (Cat. No. 15596018), Lipofectamine 3000 (Cat. No. L3000075) and enhanced chemiluminescence (ECL, Cat. No. 32106) were purchased from ThermoFisher Scientific (USA). MTT solution (Cat. No.

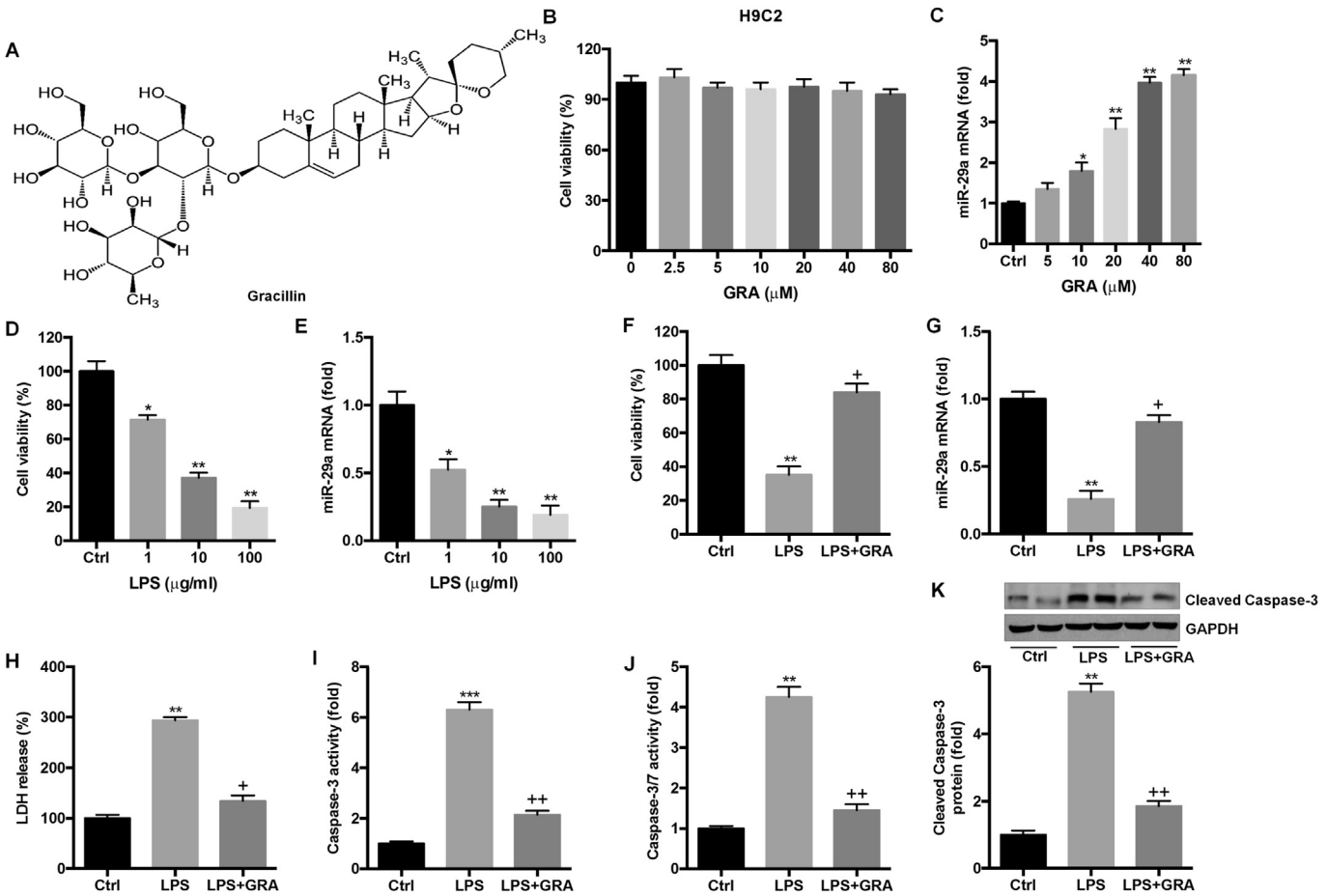

Fig. 1. Gracillin reduces apoptosis in LPS-incubated cardiomyocytes. (A) Chemical structure of Gracillin (GRA). (B) H9C2 cells were treated with GRA for 24 h, and then were collected for cell viability measurement using MTT analysis. (C) RT-qPCR analysis for miR-29a in GRA-incubated H9C2 cells for 24 h. (D) Cell viability of LPS-stimulated H9C2 cells for 24 h using MTT analysis. (E) RT-qPCR results for miR-29a in H9C2 cells treated with LPS for 24 h. (F–K) H9C2 cells were treated with LPS (10 μg/ml) for 24 h in the absence or presence of GRA. All cells were then harvested for the following assays. (F) Cell viability measurements using MTT analysis. (G) RT-qPCR assay for miR-29a in cells. (H) LDH release in cells. Measurements of (I) Caspase-3 activity and (J) Caspase-3/7 activity. (K) Cleaved Caspase-3 expression in cells was tested using Western blot. Data were presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs the Ctrl group; $^{+}p < 0.05$ and $^{++}p < 0.01$ vs the LPS group.

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C0009), LDH kit (Cat. No. C0016), RIPA lysis buffer (Cat. No. P0013B), secondary antibodies (Cat. No. A0208 and Cat. No. A0216) and DAPI (Cat. No. C1006) were purchased from Beyotime Biotechnology (Shanghai, China). Polyvinylidene fluoride (PVDF) membrane (Cat. No. IPVH85R) and ApopTag Plus Fluorescein in Situ Apoptosis Detection Kit (Cat. No. S7111) were obtained from Millipore Corporation (USA). IBL Minute TM Cytoplasmic and Nuclear Fractionation kit (Cat. No. SC-003) was purchased from Invent (USA). A Caspase-Glo 3/7 kit (Cat. No. G8090) was obtained from Promega (USA). Caspase-3/CPP32 Fluorometric Assay Kit (Cat. No. K105-25) was obtained from Biovision (USA). Primary antibodies including cleaved Caspase-3 (ab2302), phospho-IKKα (ab38515), IKKα (ab32041), IκBα (ab32518), phospho−NF−κB/p65 (ab86299) and NF-κB/p65 (ab16502) were purchased from Abcam (USA). The phospho-IκBα (PA5-36825) and GAPDH (AM4300) were obtained from Invitrogen (USA). Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594, ab150088) was purchased from Abcam. Other chemicals in this study were of analytical grade.

2.2. Cells and culture

The rat embryonic heart-derived myoblast H9C2 cells (ATCC, Manassas, USA) were incubated in DMEM (Gibco, Rockville, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin double-antibiotic in a humidified atmosphere with 5% CO_2 and 95% air at 37 °C. Gracillin (GRA) for cell treatments was purchased from Selleck (USA). The miR-Con, miR-29a mimic, scramble miR-29a and miR-29a inhibitor were transfected into H9C2 cells using Lipofectamine 3000 according to the manufacturer's guidelines. Cells were treated with the MTT solution for cell viability assay according to the manufacturer's guidelines.

2.3. Animals

All animal procedures were conducted following protocols approved by the Committee for Animal Research of First Affiliated Hospital of Xi'an Jiaotong University (Shannxi, China) and conformed to the Guide for the Care and Use of Laboratory Animals. Male, 8−10 weeks old, C57BL/6 mice (weighing 23 ± 2 g) were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China) in the study. The AAV9-miRNA-29a-EGFP and the scrambled control vectors were packed by Shanghai Obiod (Shanghai, China) and then stored at −80 °C until analysis. Preliminary tail vein injections were conducted to calculate the effects of the vectors. After injection for four weeks, cardiac samples were cut to determine the amplitude of the reporter fluorescence signal. The miR-29a levels in hearts of mice were measured to validate vector transfection. All mice were humanely treated with free access to food and water and maintained under a 12-h light/dark cycle in a constant temperature (22-25 °C). The mouse with acute heart injury was established through intraperitoneal infection with LPS at doses of 10 mg/kg. GRA was given to mice through intraperitoneal injection at a dose of 10 mg/kg per day for 7 successive days. Equal amounts of saline were used as negative control treatment. After LPS stimulation for 24 h, all mice were sacrificed, and heart tissues were collected for further analysis.

2.4. Echocardiography

After LPS challenge, animals were anaesthetized using 1.5% isoflurane and subjected to echocardiographic calculation with a MyLab 30CV ultrasound (Biosound Esaote Inc., Guangzhou, China) using a 10-MHz linear array ultrasound transducer. Cardiac parameters including fractional shortening (FS), ejection fraction (EF), LV end-diastolic diameter (LVEDd) and LV end-systolic diameter (LVEDs) were measured as previously indicated [18].

2.5. Quantitative real-time PCR

Total RNA was extracted from cells or heart samples using TRIzol following the manufacturer's instructions. The miR-29a was measured by the TaqMan® MicroRNA assay (Applied Biosystems, USA) followed the manufacturer's guidelines. U6 was used as an endogenous control. Then, cDNA was synthesized via reverse transcription reactions. RT-qPCR was conducted on an Applied Biosystems 7300 Real-Time PCR System (ThermoFisher Scientific) using the TaqMan Universal PCR Master Mix (ThermoFisher Scientific). The expression levels of each gene were quantified by the $2^{-\Delta\Delta Ct}$ method [19] and normalized to GAPDH. The primers used were shown in Supplementary Table S1.

2.6. Calculation of LDH, Caspase-3 activity and caspase-3/7 activity

The LDH, Caspase-3/7 activities and Caspase-3 activity in cells were measured following the manufacturer's descriptions.

2.7. Western blot

RIPA lysis buffer was used for total protein extraction from cells or tissues. IBL Minute TM Cytoplasmic and Nuclear Fractionation kit was used for cytoplasm and nuclei protein isolation according to the manufacturer's protocols. The isolated protein was then separated using 10−12% SDS-PAGE and electro-transferred to a PVDF membrane. Next, all membranes were blocked in 5% skimmed milk and incubated with the primary anti-bodies overnight at 4 °C. Subsequently, the membrane was incubated with the secondary antibodies at room temperature. The protein signals were detected using the ECL system. GAPDH was used to as the control antibody.

2.8. Histochemical analysis

The collected heart tissues were fixed in 4% paraformaldehyde, embedded in paraffin and cut into slices (5-μm thick). Then, the tissue sections were stained with hematoxylin & eosin (H&E) and observed under a light microscope.

2.9. Immunofluorescence

The cells were fixed with 4% paraformaldehyde at room temperature. Then, 0.03% Triton X-100 was used for membrane permeability. After blocking, the cells were incubated with primary antibody against NF-κB/p65 (1:100) at 4 °C overnight. Then, the secondary antibody conjugated to Alexa-594 was added to cells. Finally, DAPI was used for nuclei staining.

2.10. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining

Apoptosis in hearts were determined using TUNEL assay with the ApopTag Plus Fluorescein in Situ Apoptosis Detection Kit following the manufacturer's instructions.

2.11. Statistical analysis

All results were presented as mean ± SEM. The statistical analysis were conducted using GraphPad Prism (version 8, GraphPad Software Inc., USA). One-way analysis of variance (ANOVA) test was performed for the significant difference of multiple groups. The significant difference between two groups was analyzed using Student's *t*-test. Statistical significance was set at $P < 0.05$.

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3. Results

3.1. Gracillin reduces apoptosis in LPS-incubated cardiomyocytes

At first, the effects of GRA on the cytotoxicity in cardiomyocytes were measured. MTT analysis showed that GRA treatments from 2.5 to 80 μM showed no significant difference on the change of cell viability in H9C2, showing its safe use *in vitro* (Fig. 1B). Then, we found dose-dependent increase of miR-29a in GRA-incubated cardiomyocytes, especially from 10 μM of GRA treatments (Fig. 1C). LPS markedly reduced the cell viability in a dose-dependent manner (Fig. 1D). Furthermore, LPS stimulation dose-dependently reduced the expression of miR-29a in H9C2 cells (Fig. 1E). Of note, LPS-reduced cell viability in H9C2 was significantly improved by GRA co-treatment (Fig. 1F). Also, the expression of miR-29a repressed by LPS was greatly restored by GRA (Fig. 1G). LDH, indicating the condition of cellular toxicity, was detected to be highly induced by LPS, while being markedly down-regulated in GTA-treated cells (Fig. 1H). Both Caspase-3 activity and Caspase-3/-7 activity were overtly up-regulated in LPS-stimulated cardiomyocytes, whereas being significantly decreased in cells co-treated with GRA (Fig. 1I and J). Consistently, Western blot analysis verified the results that GRA obviously reduced the expression of cleaved Caspase-3 in LPS-challenged cells (Fig. 1K). Together, results above demonstrated that GRA showed protective effects against LPS-induced cell death, and could improve miR-29a expression in cardiomyocytes with or without LPS stimulation.

3.2. Gracillin alleviates cardiac injury in LPS-challenged mice

The echocardiography results suggested that LPS-challenged mice had significantly impaired cardiac function, as evidenced by the markedly reduced EF% and FS%, and the enhanced LVEDs; however, these effects were markedly reversed in GRA-treated mice (Fig. 2A). H&E staining showed that GRA treatment rescued the histological alterations in cardiac sections of LPS-treated mice (Fig. 2B). Consistently, LPS treatment caused significantly increased TUNEL-positive cells in hearts, which was apparently reduced by GRA (Fig. 2B and C). The expression of cleaved Caspase-3 was significantly decreased in hearts of LPS-challenged mice (Fig. 2D). Inflammatory response, as a key cause for acute heart injury [2,3], was then investigated. We found that the mRNA expression levels of inflammatory cytokines or chemokine, including IL-6, IL-1β, TNF-α, MCP-1 and iNOS, were significantly elevated by LPS, and these results were clearly reversed by GRA supplementation (Fig. 2E). Moreover, the activation of NF-κB signaling induced by LPS was also blunted in GRA-treated mice, as shown by the evidently reduced expression of phosphorylated IKKα, IκBα and NF-κB/p65 (Fig. 2F). Findings above indicated that GRA had protective role against LPS-induced apoptosis and inflammation in hearts.

3.3. Effects of miR-29a on apoptosis and inflammation in LPS-stimulated cardiomyocytes

Here, RT-qPCR analysis showed that miR-29a mimic dramatically up-regulated the expression level of miR-29a in H9C2 cells, whereas miR-29a inhibitor apparently down-regulated its expression compared to their corresponding control groups (Fig. 3A). Additionally, miR-29a expression level was also increased by miR-29a mimic and decreased by miR-29a inhibitor in H9C2 cells stimulated by LPS (Fig. 3B). MTT analysis showed that LPS-reduced cell viability was markedly rescued in H9C2 cells transfected with miR-29a, while being further down-regulated in H9C2 cells with miR-29a reduction (Fig. 3C). Moreover, miR-29a mimic significantly reduced LDH release in LPS-stimulated H9C2 cells; however, after transfection with miR-29a inhibitor, LPS-induced LDH releases were markedly further promoted (Fig. 3D). Then, we found that Caspase-3 activity enhanced by LPS was obviously restrained by miR-29a mimic, while being further accelerated by miR-29a inhibitor (Fig. 3E). Similar expression change of Caspase-3 cleavage was observed in H9C2 cells by Western blot (Fig. 3F). From RT-qPCR analysis, we found that LPS dramatically elevated the expression of pro-inflammatory factors including IL-6, IL-1β, TNF-α and MCP-1 compared to the Ctrl group. The increase of miR-29a significantly reduced the expression levels of these signals. In contrast, miR-29a inhibitor further enhanced the expression of IL-6, IL-1β, TNF-α and MCP-1 in LPS-stimulated H9C2 cells (Fig. 3G). Consistent with the findings of RT-qPCR, we found that the protein expression levels of NF-κB/p65 in nucleus were clearly increased in LPS/miR-29a mimic group, and decreased in LPS/miR-29a inhibitor group when compared with the LPS/NC group by immunofluorescence and Western blot assays (Fig. 3H and J). Also, the up-regulated expression of p−NF−κB in cytoplasm of LPS-treated H9C2 cells was markedly reduced by miR-29a mimic, whereas being further aggravated by miR-29a inhibitor (Fig. 3I). Therefore, miR-29a up-regulation could alleviate apoptosis and inflammation in LPS-stimulated H9C2 cells.

3.4. Gracillin alleviates LPS-induced acute heart injury through improving miR-29a expression

To further reveal the effects of miR-29a as a potential target of GRA and confirm its ability to alleviate LPS-induced cardiac injury, a rAAV9 vector with miR-29a (AAV9-miR-29a) reduction and a scrambled control vector (AAV9-Con) were packed. AAV9 vectors have been demonstrated to effectively transfect myocardial tissue and need more than 3 weeks to induce full expression in cardiac tissues. The experimental procedure was exhibited in Fig. 4A. Experimental mice were injected with viral particles containing SC or miR-29a through tail vein injection immediate after adaptation for 7 days. 3 weeks later, GRA was treated to mice, followed by LPS challenge. H&E staining showed that LPS-induced histological changes in cardiac section were obviously rescued by GRA, which were, however, markedly abrogated by miR-29a inhibitor (Fig. 4B). In addition, miR-29a inhibitor markedly abolished GRA-reduced the percentage of TUNEL-positive cells in hearts of LPS-challenged mice (Fig. 4B and C). Moreover, miR-29a inhibitor markedly restored GRA-suppressed expression of cleaved Caspase-3 in hearts of LPS-treated mice (Fig. 4D). We then found that GRA-inhibited expression levels of IL-6, IL-1β, TNF-α and p−NF−κB in cardiac tissues of LPS mice were clearly recovered by miR-29a (Fig. 4E and F). Together, the data above suggested that GRA inhibited LPS-induced acute heart injury through improving miR-29a.

4. Discussion

MiRNAs play critical role in regulating cardiovascular disease pathogenesis, such as myocarditis, myocardial infarction and heart failure [6,20]. MiR-29a could reduce cardiac fibroblasts proliferation through targeting VEGF-A/MAPK signal pathway, consequently alleviating heart damage [21]. In cardiomyocytes, miR-29a attenuated hypertrophic responses [22]. These results showed that miR-29a is related to the development of cardiac disease. In our present study, we found that LPS caused a significant reduction in miR-29a expression both in cardiomyocytes and in heart tissues, suggesting that miR-29a might be involved in the LPS-regulated acute cardiac injury. Gracillin, as a natural compound, exerts significant anti-inflammation and anti-tumor effects to control the progression of various diseases [16,17]. Here, we found that GRA markedly

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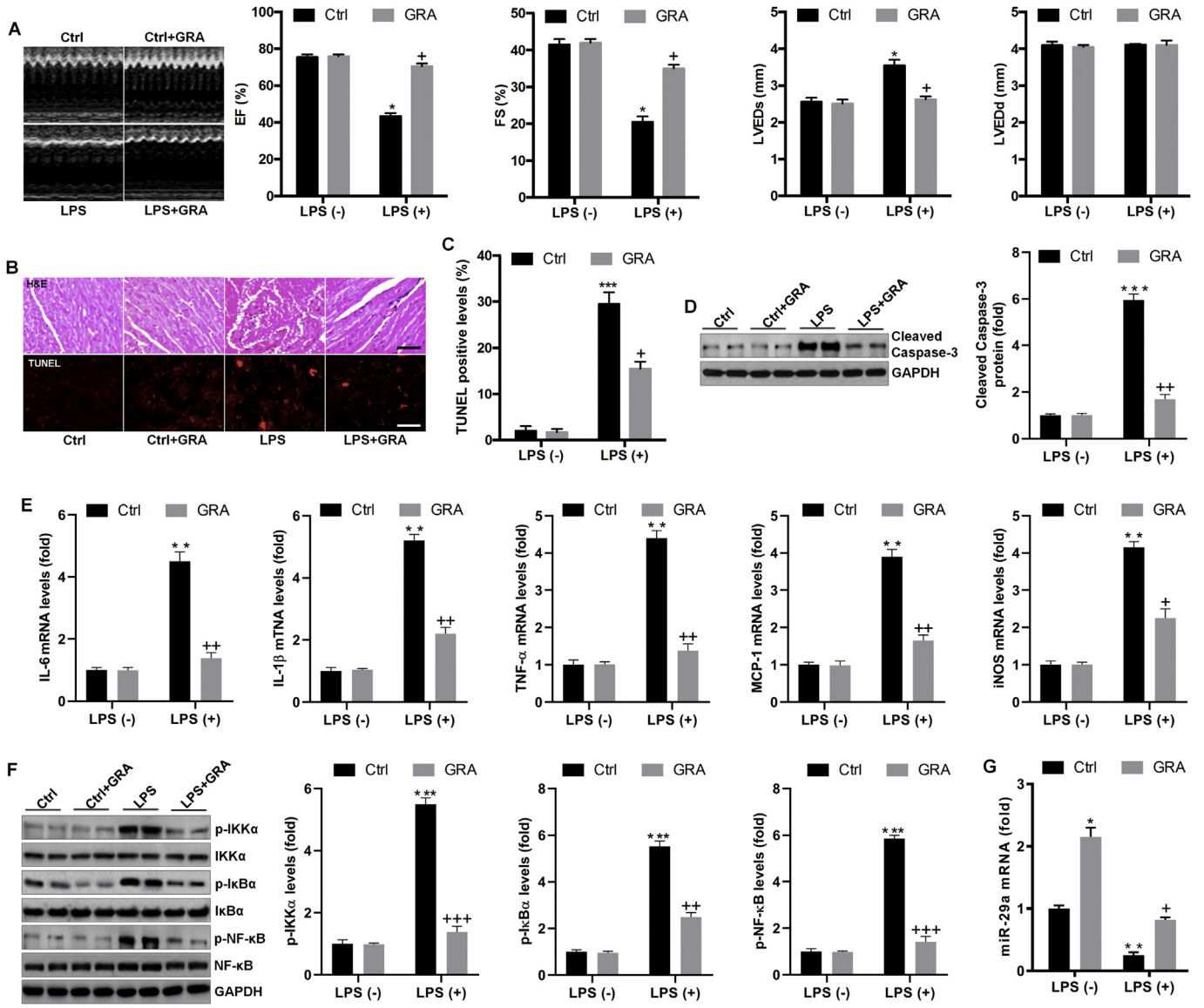

Fig. 2. Gracillin alleviates cardiac injury in LPS-challenged mice. (A) Cardiac echocardiograms were shown, and the results of EF%, FS%, LVEDs and LVEDd were quantified. (B) H&E staining (up panel) and TUNEL staining (down panel) of cardiac sections. (C) Quantification of TUNEL-positive levels in heart slides. (D) Western blotting results for cleaved Caspase-3. (E) RT-qPCR analysis of pro-inflammatory cytokines or chemokine in cardiac samples, including IL-6, IL-1β, TNF-α, MCP-1 and iNOS. (F) Western blot assays for *p*-IKKα, *p*-IκBα and p−NF−κB protein expression levels in heart tissues. (G) RT-qPCR analysis for miR-29a in hearts. Data were presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs the Ctrl group; $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs the LPS group.

stimulated the expression of miR-29a in H9C2 cells and in cardiac tissues. In addition, LPS-reduced miR-29a was greatly rescued by GRA *in vitro* and *in vivo*. We also observed that LPS-induced cardiac dysfunction, apoptosis and inflammatory response were obviously reversed by GRA administration, which were largely associated with the improvement of miR-29a expression. Therefore, findings above for the first time suggested that GRA might be a promising therapeutic strategy for sepsis-induced cardiac injury.

Accumulating studies have showed that apoptosis is a pivotal process during sepsis. Suppressing apoptosis by various kinds of agents is efficient to alleviate sepsis-induced myocardial disease [23]. Several studies have showed that LPS directly induced apoptosis in cardiomyocyte through the activation of Caspase-3 [24,25]. GRA has been demonstrated to regulate apoptotic response in tumor cells, which is also related to the expression change of Caspase-3 [17]. In our study, we found that GRA could inhibit apoptosis in LPS-stimulated cardiomyocytes and in hearts from LPS-challenged mice, consequently alleviating acute heart injury. Overexpression of miR-29a suppressed the apoptosis of renal tubular epithelial cells, which was involved in the alleviation of renal damage [26]. In this work, the *in vitro* results showed that LPS-induced apoptosis in H9C2 cells could be repressed by miR-29a mimic, while being further accelerated by miT-29a inhibitor, indicating the anti-apoptosis activity of miR-29a during acute cardiac injury. However, there are also studies reporting that miR-29a induced apoptosis in rheumatoid arthritis fibroblast-like synoviocytes [27]. The up-regulation of miR-29a in high glucose-incubated myocardial cells was observed, which was accompanied with elevated cellular apoptosis [28]. Therefore, the effects of miR-29a on the regulation of apoptosis are controversial, which might be associated with the cell types stimulated under different stresses. As for this further studies are still required in future to better understanding the action mechanisms of miR-29a.

Cardiomyocytes, once being stimulated by LPS, produce a large

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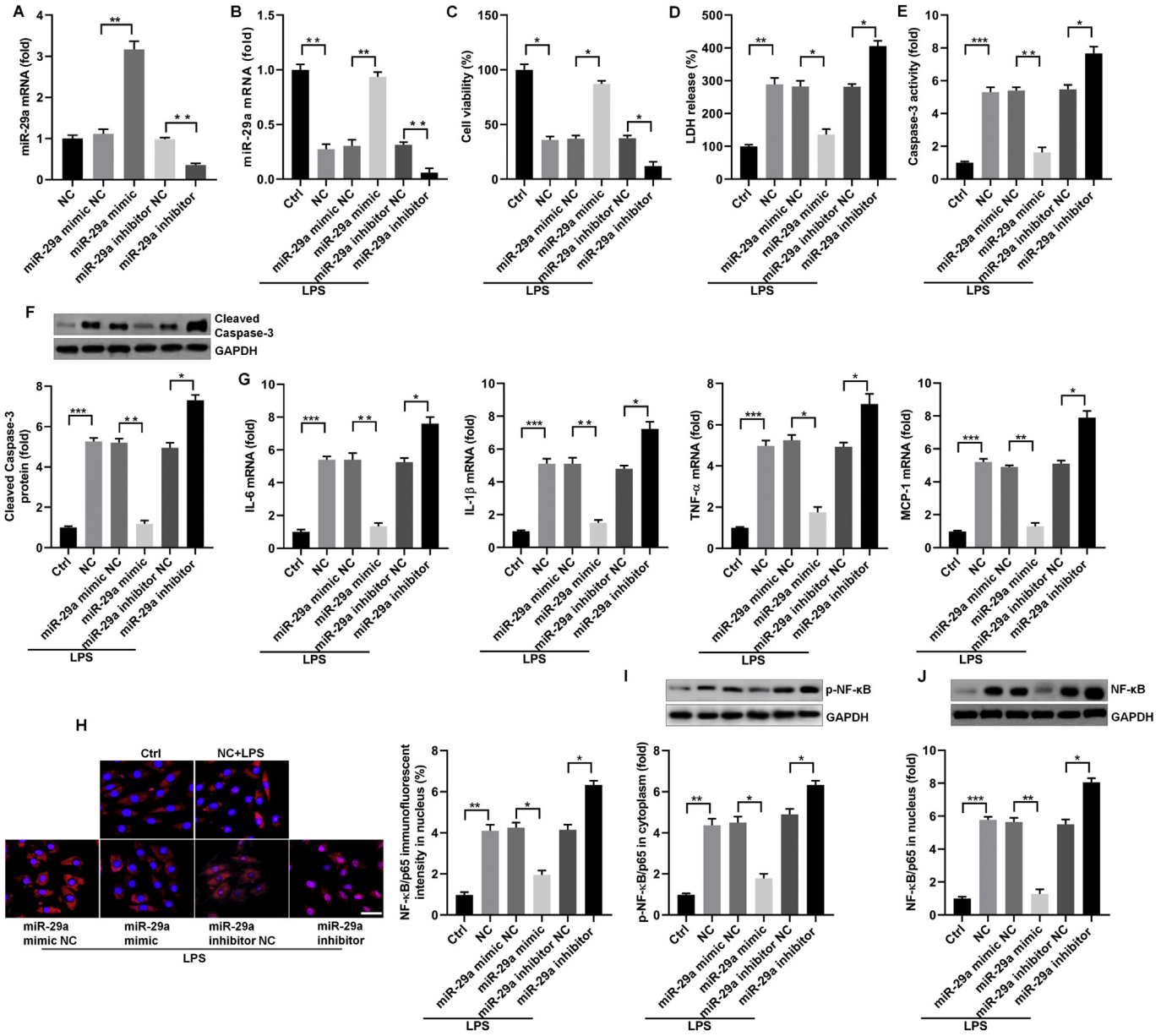

Fig. 3. Effects of miR-29a on apoptosis and inflammation in LPS-stimulated cardiomyocytes. (A) H9C2 cells were transfected with mi-29a mimic, miR-29a inhibitor or their corresponding negative control (NC) for 24 h. Then, all cells were collected for RT-qPCR analysis of miR-29a. (B–J) H9C2 cells were transfected with mi-29a mimic, miR-29a inhibitor or their corresponding negative control for 24 h, followed by LPS (10 μg/ml) stimulation for another 24 h. Subsequently, all cells were harvested for further analysis. (B) RT-qPCR results for miR-29a. (C) MTT assays for cell viability. (D) LDH release in cells was assessed. (E) Caspase-3 activity was measured. (F) Cleaved Caspase-3 protein levels were evaluated. (G) RT-qPCR analysis for IL-6, IL-1β, TNF-α and MCP-1. (H) Immunofluorescence for NF-κB/p65 in cells. Protein expression levels of (I) p–NF–κB and (J) NF-κB in cytoplasm and nucleus, respectively, were determined by western blotting assays. Data were presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

number of proinflammatory cytokines or chemokine, such as IL-1β, TNF-α, IL-6, MCP1 and iNOS, resulting in cardiac injury [29]. In accordance with previous findings, our results confirmed that the mRNA levels of proinflammatory factors (TNFα, IL-1β, IL-6, MCP1 and iNOS) were markedly higher after LPS treatment in cells and in heart tissues. It is generally accepted that the NF-κB signaling plays a crucial role in regulating series of cardiac inflammatory responses triggered by LPS [30,31]. We here found that found that LPS induced NF-κB activation as well as IKKα and IκBα phosphorylation in cardiomyocytes and/or heart tissues, all of which were markedly restrained by GTA treatment. GRA was found to effectively attenuate skin injury by reducing inflammatory response [16]. Herein, we supposed that GRA-alleviated acute heart injury by LPS was also attributed to the suppression of inflammation through reducing the translocation of NF-κB into nucleus, contributing to the decreases in the secretion of proinflammatory factors. LPS stimulation of macrophages gradually enhanced the expression of miR-29a, and this process was dependent on the LPS concentrations. Promoting miR-29a in macrophages elevated the expression of proinflammatory cytokines such as IL-1β and IL-6 [32]. Increased expression of miR-29a in extracellular vesicle was responsible for chronic inflammation and synaptic injury in neurons [33]. However, in thioacetamide-treated mice with hepatic injury, miR-29a suppressed liver inflammation [34]. In our study, LPS-induced inflammation and NF-κB activation were markedly alleviated by miR-29a mimic, while being exacerbated in H9C2 cells transfected

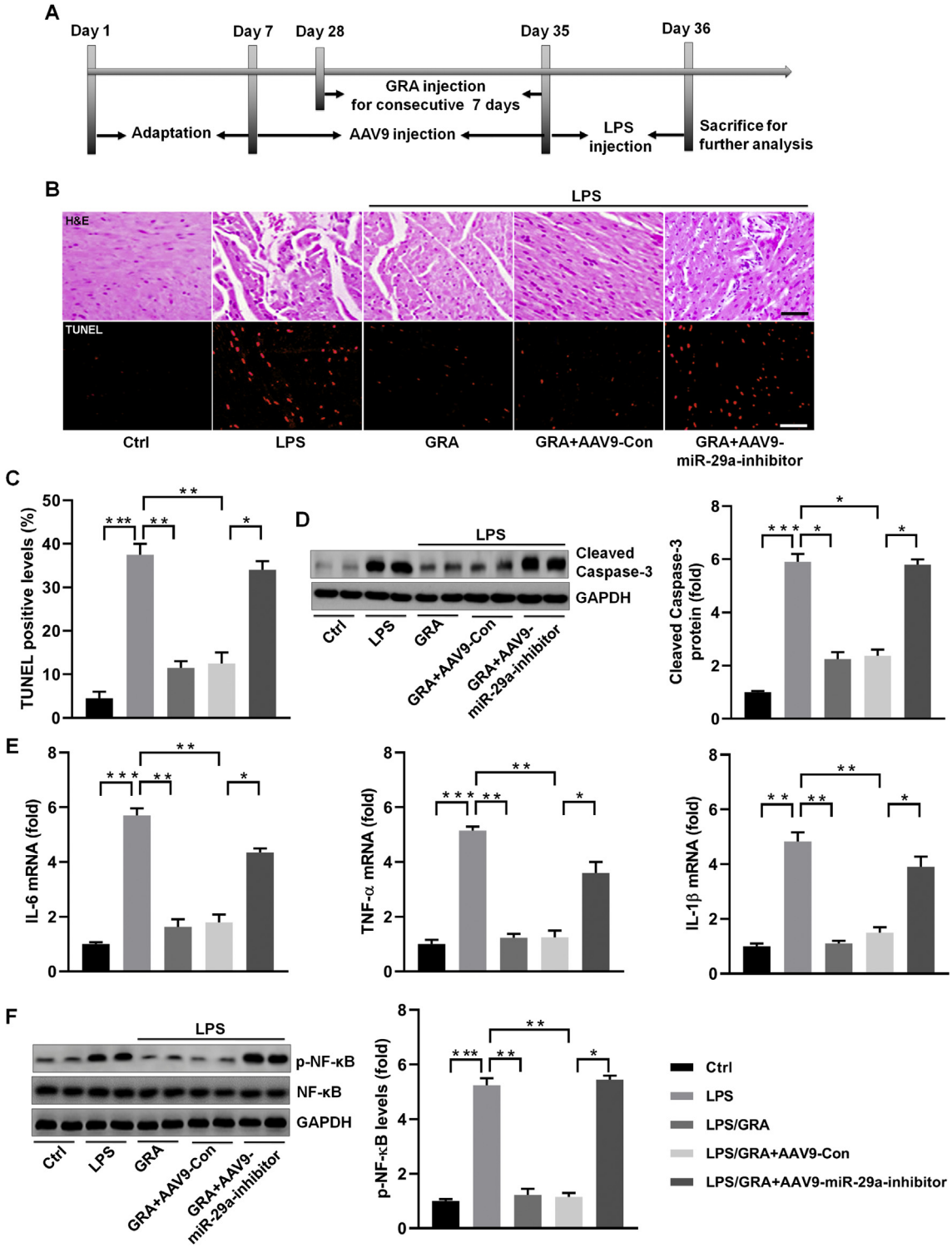

Fig. 4. Gracillin alleviates LPS-induced acute heart injury through improving miR-29a expression. (A) Procedure for experiment *in vivo*. (B) H&E staining (up panel) and TUNEL staining (down panel) of cardiac sections. (C) Quantification of TUNEL-positive levels in hearts. (D) Western blotting results for cleaved Caspase-3. (E) RT-qPCR analysis for IL-6, IL-1β and TNF-α in hearts. (F) Western blot analysis for p−NF−κB in hearts. Data were presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

with miR-29a inhibitor. These results demonstrated that miR-29a might exhibit a protective role in LPS-damaged cardiomyocytes. Our *in vivo* experiments also verified that miR-29a inhibitor significantly abrogated the protective role of GRA against LPS-triggered heart injury also through reducing apoptosis and inflammatory response. Nevertheless, given the dual function of miR-29a in regulating inflammation, more experiments are still warranted to identify the precious effects of miR-29a on LPS-induced cardiac injury.

Taken together, these results from our present study demonstrated that GRA-regulated increase of miR-29a contributed to the suppression of apoptosis and inflammatory response, which

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consequently improved LPS-induced acute heart injury. Thus, GRA might be a promising therapy to protect sepsis-associated heart diseases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.11.129.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.11.129.

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