International Journal of Biochemistry and Cell Biology 109 (2019) 8–16

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Gastrodin protects rat cardiomyocytes H9c2 from hypoxia-induced injury by up-regulation of microRNA-21

Yu Xing*, Ling Li

Department of Cardiology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, China

ARTICLE INFO

Keywords:
Myocardial infarction
Hypoxic injury
Gastrodin
microRNA-21
PTEN/PI3K/AKT/NF-κB

ABSTRACT

Background: The *in vivo* protective role of gastrodin (GSTD) in myocardial infarction (MI) has been reported. However, the underlying mechanism remains unclear. Herein, we aimed to explore the effects of GSTD on hypoxia-injured H9c2 cells as well as the downstream microRNAs (miRNAs) and signaling cascades.

Methods: Hypoxic injury model was constructed to mimic MI. Effects of GSTD pretreatment on cell proliferation and apoptosis were measured by CCK-8 assay, Western blot analysis, and flow cytometry/Western blot analysis, respectively. Expression of miR-21 in cells treated with hypoxia or hypoxia plus GSTD was determined by stem-loop RT-PCR. Whether GSTD affected hypoxia-injured cells via miR-21 was subsequently verified. The direct target of miR-21 was studied by bio-informatics ways and luciferase reporter assay. Besides, expression levels of key kinases in the PTEN/PI3K/AKT and NF-κB pathways were testified by Western blot analysis.

Results: Hypoxia-induced decrease of cell viability, up-regulation of p53 and p16, increase of apoptotic cells and up-regulation of Bax, cleaved casapse-3 and cleaved caspase-9 were all mitigated by GSTD pretreatment. Expression of miR-21 was up-regulated by hypoxia and was further up-regulated by GSTD treatment. In transfected H9c2 cells, effects of GSTD on hypoxia-treated cells were augmented by miR-21 overexpression while were reversed by miR-21 inhibition. PDCD4 was confirmed as a direct target of miR-21, and reversed the effect of miR-21 on hypoxia-injured cells. Finally, GSTD down-regulated PTEN expression and enhanced phosphorylation levels of PI3K, AKT, p65 and IκBα via up-regulating miR-21.

Conclusion: GSTD attenuated hypoxic injury of H9c2 cells through activating PTEN/PI3K/AKT and NF-κB pathways via up-regulating miR-21.

1. Introduction

Myocardial infarction (MI) is a common ischemic heart disease affecting nearly 8 million people in the United States (Mozaffarian et al., 2015). Diverse local changes are observed after MI, for instance, ventricular remodeling, alteration of ventricular wall structure, and formation of non-contractile scar tissues, resulting in cardiac dysfunction (Grans et al., 2014, Le et al., 2018). Outcome of MI has been improved with reduced mortality rates owing to the advance of modern medicine (Le LV et al., 2018). However, as a global problem, MI remains to be a leading cause of morbidity and mortality worldwide (DeFilippis et al., 2017). Innovative therapeutic drugs for restoration and regeneration after MI are urgently needed.

MI is defined as the cardiomyocyte death caused by prolonging ischemia (lack of oxygen and nutrient supply) (Feriani et al., 2018). Hypoxic injury after MI is a major cause of irreversible loss of cardiomyocytes (Chen-Scarabelli et al., 2012). A large body of studies have explored the potential therapeutic strategies for MI through experiments performed in hypoxia-treated H9c2 cells, a cell line derived from rat embryonic ventricular cardiomyocytes with many similarities to primary cardiomyocytes (Duan et al., 2016; Liu et al., 2018). In adults, cardiomyocytes possess limited proliferative capacity, making induction of proliferation in cardiomyocytes become a possible strategy for therapeutic cardiac regeneration (Boon and Dimmeler, 2015). Therefore, alteration of proliferation and apoptosis in hypoxia-treated H9c2 cells can be used as an evaluation index for potential therapeutic drugs.

Gastrodin (GSTD) is a phenolic glucoside extracted from the rhizome of *Gastrodiaelata elata* Blume, and its chemical structure was shown as Fig. 1A. As the principal bioactive component of *Gastrodiaelata elata* Blume, GSTD possesses various pharmacological functions. GSTD has reported as a drug candidate in neurodegenerative disease due to the anti-oxidative activity (Zhang et al., 2016). Cerebral ischemic damage in mice can be alleviated by GSTD owing to the antioxidant, anti-inflammatory and anti-apoptotic activities (Peng et al.,

* Corresponding author at: Department of Cardiology, The First Affiliated Hospital of Zhengzhou University, No.1, Jianshe East Road, Zhengzhou, 450052, China.
E-mail address: xingyu1232018@sina.com (Y. Xing).

https://doi.org/10.1016/j.biocel.2019.01.013
Received 5 September 2018; Received in revised form 21 December 2018; Accepted 21 January 2019
Available online 23 January 2019
1357-2725/

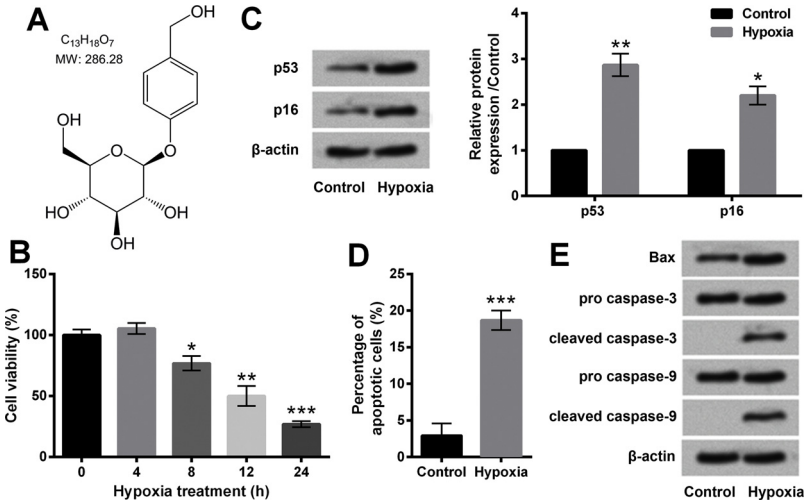

Fig. 1. Hypoxia induced H9c2 cell injury. (A) Chemical structure of gastrodin. H9c2 cells were incubated under hypoxia for 4, 8, 12 and 24 h, and cells incubated under normoxia were acted as control. (B) Cell viability was determined by CCK-8 assay. H9c2 cells were incubated under hypoxia for 12 h, and cells incubated under normoxia were acted as control. (C) Expression of p53 and p16 was evaluated by Western blot analysis. (D) Percentage of apoptotic cells was quantified by flow cytometry assay. (E) Expression of proteins associated with apoptosis was measured by Western blot analysis. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

2015). The protective role of GSTD in myocardial cells of hypoxia/reoxygenation-injured rats was reported previously (Li et al., 2018). A study has reported that GSTD suppresses inflammatory response in lipopolysaccharide-treated H9c2 cells (Yang et al., 2013). However, the specific roles of GSTD in hypoxia-treated H9c2 cells as well as the molecular mechanism are poorly studied.

MicroRNAs (miRNAs/miRs), the endogenous non-coding RNAs with 22 nucleotides in length, which have been identified to control important processes involved in the pathophysiological consequences of MI (Bartel, 2004; Boon and Dimmeler, 2015). Currently, several potential drugs extracted from herbs have been proven to exert their pharmacological activities via regulation of miRNAs (Gu et al., 2016; Wu et al., 2018). MiR-21 is a small multi-faceted RNA, which plays a pivotal role in the pathogenesis and progression of various cancers (Song et al., 2016; Zhang et al., 2008). Additionally, recent study revealed that miR-21 could promote cardiac fibrosis after MI by regulating Smad7 (Yuan et al., 2017). Evidence from Gu et al. demonstrated that serum-derived extracellular vesicles exerted the protective effect against acute MI by mediating miR-21 (Gu et al., 2018). However, whether miR-21 is involved in regulating the functions of GSTD in MI remains to be poorly understood.

PTEN/PI3K/AKT and NF-κB signaling pathways participate in regulating various biological processes, which functions in MI have been revealed in recent studies. It has reported that pharmacological inhibition of PTEN can limit MI size and improve left ventricular function post-infarction (Keyes et al., 2010). An interesting study displayed that Acacetin could alleviate cardiac remodeling after MI by regulating PI3K/AKT pathway (Chang et al., 2017). Blockade of NF-κB has been reported to improve cardiac dysfunction and survival after MI (Onai et al., 2007). However, relatively little is known about mechanisms regulating PTEN/PI3K/AKT and NF-κB signaling pathways in MI. Herein, we constructed hypoxic injury model using H9c2 cells to mimic MI progression *in vitro*. Effects of GSTD on proliferation and apoptosis in hypoxia-treated H9c2 cells were testified to explore the possible effects of GSTD on MI. In the meantime, we also explored the possibly regulatory mechanism of GSTD in hypoxia-treated H9c2 cells through investigating miR-21 and PTEN/PI3K/AKT and NF-κB signaling cascades.

2. Materials and methods

2.1. Cell culture and treatment

H9c2 cell line (ATCC® CRL-1446™) was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Complete culture medium was consisted of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) and 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA). Normally, cells were grown in a humidified incubator filled with 95% air and 5% CO_2 at 37 °C. For hypoxia treatment, cells were incubated in a hypoxic incubator filled with 94% N_2, 5% CO_2, and 1% O_2 at 37 °C.

GSTD (purity ≥ 98%, CAS Number 62499-27-8), purchased from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in DMEM to 10 mM as stock solution. The stock solution was diluted in DMEM, and the final concentrations of GSTD were 0 (Control), 1, 5, and 10 μM. Cells were pre-treated by GSTD for 2 h before hypoxia treatment.

2.2. Cell transfection

miR-21 mimic, miR-21 inhibitor, and their negative control (NC) were synthesized by GenePharma Co. (Shanghai, China). The full-length of programmed cell death 4 (PDCD4) sequence was constructed in pcDNA3.1 plasmid (pcDNA-PDCD4). The empty pcDNA3.1 plasmid served as a control group. The plasmids were respectively transfected into H9c2 cells using the Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol.

2.3. Cell viability assay

Cell viability of treated H9c2 cells was measured by using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Transfected or untransfected cells were seeded in 96-well plates at 5×10^3 cells/well. After incubation at 37 °C overnight, cells were treated with hypoxia and/or GSTD pretreatment. Then, the culture medium was replaced by DMEM containing 10% CCK-8 solution. After incubation at 37 °C for 1 h, the 96-well plates were subjected into a Microplate Reader (Bio-Rad, Hercules, CA, USA) to detect the absorbance at 450 nm.

2.4. Apoptosis assay

Cell apoptosis of treated H9c2 cells was analyzed after double-staining with FITC Annexin V and propidium iodide (PI). Transfected or untransfected cells were seeded in 6-well plates at 1×10^5 cells/well. After incubation at 37 °C overnight, cells were treated with hypoxia and/or GSTD pretreatment. Subsequently, cells were collected and washed in cold PBS. Cells (1×10^5) were suspended in 100 μL 1× Binding Buffer, followed by staining with 5 μL FITC Annexin V and 5 μL PI, provided by the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, Franklin Lakes, NJ, USA), at room temperature for 15 min in the dark. Then, 400 μL 1× Binding Buffer was added, and stained cells were analyzed by using a flow cytometer (Beckman Coulter Inc., Miami, FL, USA). Percentage of apoptotic cells was evaluated by FlowJo

software (Tree Star, San Carlos, CA, USA).

2.5. Dual luciferase reporter assay

The 3′untranslated region (3′UTR) target site was generated by PCR and the luciferase reporter constructs with the PDCD4 3′UTR carrying a putative miR-21-binding site into pMiR-report vector were amplified by PCR. The plasmids were co-transfected with miR-21 mimic or the control into H9c2 cells using Lipofectamine 3000 (Invitrogen). After transfection, reporter assays were done using the dual-luciferase assay system (Promega, Fitchburg, WI, USA) according to the manufacturer's information.

2.6. Stem-loop RT-PCR

Total RNA was isolated from H9c2 cells using Trizol reagent (Invitrogen) as suggested by the manufacturer. RNA samples (500 ng) were reverse transcribed into cDNA using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The thermocycling program of reverse transcription was 30 min at 16 °C, 30 min at 42 °C and 5 min at 85 °C. The expression level of miR-21 was measured against an endogenous control (U6) by real-time PCR using the Taqman® Universal Master Mix II (Applied Biosystems) and the 7500 Fast Real-Time PCR System (Applied Biosystems), as suggested by the manufacturer. The thermal cycling conditions were 1 cycle at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative expression fold was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Western blot analysis

Proteins of treated H9c2 cells were extracted using RIPA buffer (Beyotime, Shanghai, China) supplemented with 1 mM PMSF (Beyotime). The BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was used to evaluate the protein concentration of protein samples. Then, protein extracts were resolved on SDS-PAGE and electro-transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membranes were probed with specific primary antibodies against p53 (ab131442), p16 (ab51243), Bax (ab53154), PDCD4 (ab80590), PTEN (ab32199), PI3K (ab191606), phospho (p)-PI3K (ab182651), AKT (ab8805), p-AKT (ab38449), p65 (ab16502), p-p65 (ab28856), β-actin (ab8227, all Abcam, Cambridge, UK), caspase-3 (#9662), caspase-9 (#9508), IκBα (#4812) or p-IκBα (#2859, all Cell Signaling Technology, Beverly, MA, USA). PVDF membranes were washed by Tris-buffered saline containing 1% Tween-20 (TBST) and were incubated with goat anti-rabbit IgG-HRP (ab205718) or goat anti-mouse IgG-HRP (ab6789). After washing with TBST, the immunoreactive signals were detected using a SuperSignal West Pico chemiluminescence ECL kit (Pierce) and the intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

2.8. Statistical analysis

Experiments were performed in triplicate with three repeats. Data were presented as the mean ± standard deviation (SD). Statistical analysis was performed using Graphpad Prism 5 software (GraphPad, San Diego, CA, USA) and statistical comparisons were performed by one-way analysis of variance (ANOVA) with Dunnett's post hoc test or two-tailed Student's *t*-test. *P*-values < 0.05 were deemed significant.

3. Results

3.1. Hypoxia induced hypoxic injury in H9c2 cells

Effects of hypoxia on cell viability, proliferation and apoptosis were analyzed to evaluate whether hypoxic injury model was constructed successfully. CCK-8 results in Fig. 1B showed cell viability was significantly reduced by hypoxia treatment for 8 h ($P < 0.05$), 12 h ($P < 0.01$) and 24 h ($P < 0.001$) relative to the cells incubated under normoxia. Cell viability stayed unchanged when the duration of hypoxia treatment was 4 h. Since cell viability was reduced to a half when the duration of hypoxia treatment was 12 h, therefore, cells were incubated under hypoxia for 12 h in the subsequent experiments. In Fig. 1C, expression of proteins associated with proliferation, including p53 and p16, was prominently up-regulated after hypoxia treatment as relative to the control group ($P < 0.05$ or $P < 0.01$). Flow cytometry assay showed percentage of apoptotic cells in the hypoxia group was dramatically higher than that in the control group ($P < 0.001$, Fig. 1D). Likewise, expression of pro-apoptotic Bax, cleaved caspase-3 and cleaved caspase-9 was observably up-regulated by hypoxia treatment (Fig. 1E). Results illustrated that H9c2 hypoxic injury was induced successfully after hypoxia treatment.

3.2. GSTD pretreatment attenuated hypoxia-induced H9c2 cell injury

Effects of GSTD on hypoxic injury of H9c2 cells were subsequently explored. To determine the adequate dosage of GSTD, cells were incubated with 1, 5, and 10 μM GSTD and cell viability was assessed. In

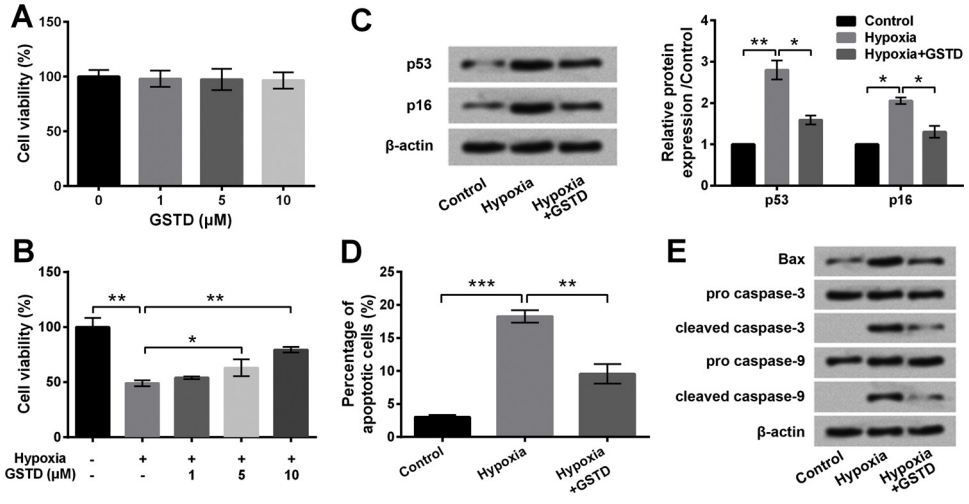

Fig. 2. Gastrodin (GSTD) attenuated hypoxia-induced H9c2 cell injury. H9c2 cells were stimulated with 1, 5 or 10 μM GSTD for 24 h, and non-treated cells were acted as control. (A) Cell viability was determined by CCK-8 assay. H9c2 cells were stimulated with 0, 1, 5 or 10 μM GSTD for 2 h, followed by incubation under hypoxia for 12 h. Non-treated cells were acted as control. (B) Cell viability was testified by CCK-8 assay. H9c2 cells were stimulated with 0 or 10 μM GSTD for 2 h, followed by incubation under hypoxia for 12 h. Non-treated cells were acted as control. (C) Expression of p53 and p16 was evaluated by Western blot analysis. (D) Percentage of apoptotic cells was quantified by flow cytometry assay. (E) Expression of proteins associated with apoptosis was measured by Western blot analysis. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

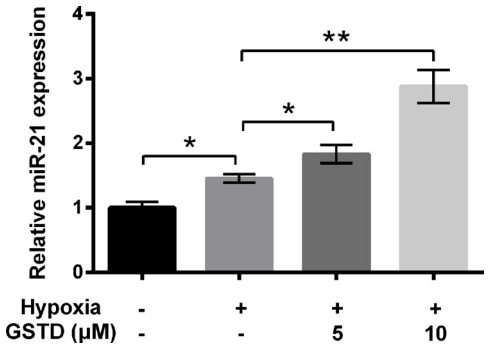

Fig. 3. Gastrodin (GSTD) up-regulated miR-21 expression in H9c2 cells. H9c2 cells were stimulated with 0, 5 or 10 μM GSTD for 2 h, followed by incubation under hypoxia for 12 h. Non-treated cells were acted as control. Expression of miR-21 was determined by stem-loop RT-PCR. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

Fig. 2A, there was no statistical difference of cell viability when the dosage of GSTD increased. That is to say, 1–10 μM GSTD showed neither pro- nor anti-survival effects on H9c2 cells under normoxia. Then, cells were stimulated with GSTD (1, 5 or 10 μM), followed by hypoxia treatment. CCK-8 assay showed 5–10 μM GSTD notably elevated cell viability compared with the hypoxia-treated cells ($P < 0.05$ or $P < 0.01$, Fig. 2B). The dosage of GSTD for subsequent experiments was 10 μM due to the stronger pro-survival effects of 10 μM GSTD on hypoxia-treated cells. Afterwards, we found expression of p53 and p16 was down-regulated by GSTD as relative to the hypoxia group (both $P < 0.05$, Fig. 2C). In the meantime, percentage of apoptotic cells as well as expression levels of Bax, cleaved caspase-3 and cleaved caspase-9 was distinctly lowered by GSTD pretreatment ($P < 0.01$, Fig. 2D-2E). Results collectively illustrated that hypoxic injury in H9c2 cells was attenuated by GSTD pretreatment.

3.3. GSTD treatment up-regulated miR-21 expression in H9c2 cells

The possible relationship between GSTD and miR-21 was explored. Compared with cells incubated under normoxia, miR-21 level in hypoxia-treated cells was markedly higher ($P < 0.05$, Fig. 3). In cells pretreated with GSTD (5 or 10 μM), miR-21 levels were significantly enhanced compared with the hypoxia group ($P < 0.05$ or $P < 0.01$). Results illustrated that GSTD could up-regulate miR-21 level in hypoxia-treated H9c2 cells.

3.4. GSTD affected hypoxia-treated H9c2 cells through up-regulating miR-21

Since the miR-21 level was altered by GSTD, we hypothesized that GSTD might affect hypoxia-treated cells through up-regulating miR-21 and the following experiments were performed to verify the hypothesis. As evidenced by Fig. 4A, miR-21 levels in cells transfected with miR-21 mimic were remarkably higher ($P < 0.001$) whereas those in cells transfected with miR-21 inhibitor were markedly lower ($P < 0.01$), as compared to the NC-transfected cells. Results indicated that transfection with miRNAs could dysregulate miR-21 expression successfully. The results in Fig. 4B revealed that miR-21 overexpression significantly promoted cell viability as compared to NC group ($P < 0.05$). However, miR-21 overexpression has no obvious effect on cell apoptosis and apoptosis-associated factors (Fig. 4C and 4D). Then, these transfected cells were treated with GSTD and hypoxia, and miR-21 expression level, cell viability, proliferation and apoptosis were testified. As shown in Fig. 5A, the expression level of miR-21 was up-regulated by miR-21 overexpression in GSTD and hypoxia treated cells ($P < 0.001$). Additionally, we found the effects of GSTD on hypoxia-treated cells were further enhanced by miR-21 overexpression, as miR-21 overexpression significantly increased cell viability ($P < 0.05$, Fig. 5B), reduced levels of p53 and p16 (both $P < 0.05$, Fig. 5C), lowered percentage of apoptotic cells ($P < 0.05$, Fig. 5D), and down-regulated expression of pro-apoptotic proteins (Fig. 5E), compared with the hypoxia + GSTD + NC group. Moreover, effects of miR-21 inhibition on cells treated with hypoxia and GSTD were opposite to those of miR-21 overexpression. Results talked above illustrated that GSTD might affect hypoxia-treated H9c2 cells through up-regulating miR-21 expression.

3.5. PDCD4 was a direct target of miR-21

To make a linkage between GSTD and miR-21, the target gene of miR-21 was explored. The bioinformatics software of TargetScan (http://www.targetscan.org/) and microRNA database (http://www.microrna.org/) predicated a conserved target-site for rno-miR-21-5p within the PDCD4 3′-UTR at 281–288 nucleotides (Fig. 6A). The protein and mRNA expression levels of PDCD4 were significantly down-regulated in miR-21 mimic-transfected cells, while up-regulated in miR-21 inhibitor-transfected cells, indicating a negative regulation between PDCD4 and miR-21 ($P < 0.01$, Fig. 6B). Subsequently, dual luciferase reporter assay showed that miR-21 overexpression led to a significant decrease of reporter activity in PDCD4-WT transfected H9c2 cells ($P < 0.05$), but did not affect the reporter activity in PDCD4-MUT transfected cells (Fig. 6C). Results talked above suggested that PDCD4 was a direct target of miR-21.

3.6. PDCD4 reversed the effect of miR-21 on hypoxia-treated H9c2 cells

To further explore the involvement of PDCD4 in mediating the effect of miR-21 on hypoxia-treated H9c2 cells, the plasmids of pcDNA-PDCD4 and its control (pcDNA3.1) were transfected in H9c2 cells. As shown in Fig. 7A, the protein and mRNA levels of PDCD4 were all up-regulated in pcDNA-PDCD4 transfected cells as compared to that in pcDNA-3.1 transfected cells ($P < 0.001$). The functional experiments revealed that miR-21 overexpression increased cell viability ($P < 0.05$, Fig. 7B), decreased levels of p53 and p16 (both $P < 0.05$, Fig. 7C), reduced cell apoptosis ($P < 0.05$, Fig. 7D), and down-regulated the protein levels of Bax, cleaved caspase-3 and cleaved caspase-9 (Fig. 7E) in hypoxia-treated cells. However, effects of miR-21 overexpression on cells treated with hypoxia were obviously reversed by PDCD4 overexpression ($P < 0.05$ or $P < 0.01$, Fig. 7B-7D). Results talked above hinted that PDCD4 reversed the effect of miR-21 on hypoxia-treated H9c2 cells, which might be an important regulator in MI.

3.7. GSTD activated the PTEN/PI3K/AKT and NF-κB pathways via miR-21 in H9c2 cells

The possible involvements of the PTEN/PI3K/AKT and NF-κB pathways in GSTD-associated modulation were finally assessed. In Fig. 8A, hypoxia treatment obviously up-regulated PTEN expression ($P < 0.01$) while decreased phosphorylation levels of PI3K and AKT (both $P < 0.05$). Those effects of hypoxia were observably reversed by GSTD pretreatment (all $P < 0.05$). Moreover, the effects of GSTD on expression of those key kinases were significantly augmented by miR-21 overexpression while were reversed by miR-21 silence (all $P < 0.05$). In Fig. 8B, phosphorylation levels of p65 and IκBα were prominently increased by GSTD pretreatment (both $P < 0.05$), and were further increased in miR-21-overexpressed cells with GSTD pretreatment (both $P < 0.05$). Effects of GSTD pretreatment on phosphorylation of key kinases in the NF-κB pathway were reversed by miR-21 inhibition (both $P < 0.05$). Results talked above indicated that GSTD could activate the PTEN/PI3K/AKT and NF-κB pathways via regulating miR-21 in H9c2 cells.

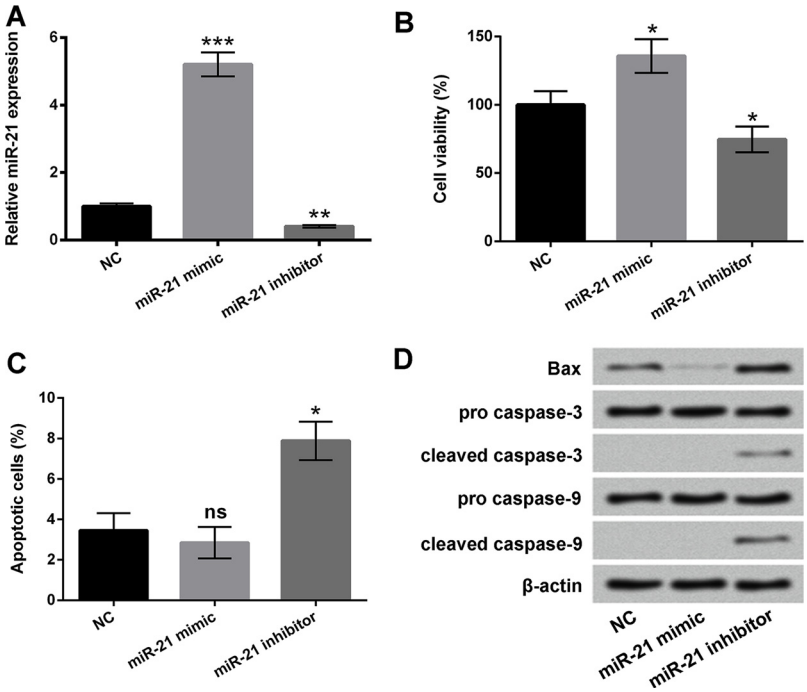

Fig. 4. MiR-21 promoted cell viability, but had no effect on cell apoptosis in H9c2 cells. H9c2 cells were transfected with miR-21 mimic, miR-21 inhibitor or their negative control (NC), and NC-transfected cells were acted as control. (A) Expression of miR-21 was determined by stem-loop RT-PCR. (B) Cell viability was detected by CCK-8 assay. (C) Percentage of apoptotic cells was quantified by flow cytometry assay. (D) Expression of proteins associated with apoptosis was measured by Western blot analysis. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4. Discussion

Despite the reported protective role of GSTD in cardiomyocytes from hypoxia/reoxygenation-injured rats, the specific effects of GSTD on hypoxic injury of H9c2 cells as well as the underlying mechanisms remain unclear. In the present study, we found that GSTD pretreatment could effectively attenuate hypoxia-induced H9c2 cell injury. Stem-loop RT-PCR results showed that miR-21 expression, up-regulated by hypoxia, was further up-regulated by GSTD in hypoxia-injured cells. More experiments verified that effects of GSTD on hypoxia-injured cells were augmented by miR-21 overexpression while were reversed by miR-21 inhibition. PDCD4 was verified to be a direct target of miR-21, and the effect of miR-21 on hypoxia-injured H9c2 cells was reversed by PDCD4. Besides, the PTEN/PI3K/AKT and NF-κB pathways were activated by GSTD, possibly by up-regulation of miR-21.

First of all, proliferation and apoptosis of H9c2 cells were assessed to evaluate whether a hypoxic injury model was constructed successfully. As a cyclin-dependent kinase (CDK) inhibitor, p16 binds to CDK4/CDK6 and represses formation of cyclinD1-CDK4/6 complex, leading to inhibited proliferation (Romagosa et al., 2011). p53 which can be stabilized and activated quickly in response to hypoxia is identified to be a tumor suppressor, showing anti-proliferative and death-promoting effects (Brady and Attardi, 2010; Kruiswijk et al., 2015). In our study, cell viability was reduced along with up-regulation of p16 and p53 after hypoxia treatment, indicating that hypoxia treatment repressed proliferation of H9c2 cells. Mitochondria, as the major consumers of oxygen, is sensitive to hypoxia, and they may release pro-apoptotic proteins in response to hypoxia, leading to activation of caspases (Fuhrmann and Brune, 2017; Thornton and Hagberg, 2015). In our study, the elevated apoptotic cells as well as up-regulation of pro-apoptotic Bax and activation of caspases indicated that hypoxia treatment promoted H9c2 cell apoptosis. The repressed proliferation and enhanced apoptosis in H9c2 cells illustrated that hypoxic injury model was constructed successfully.

Subsequently, the effects of GSTD pretreatment on hypoxic injury of H9c2 cells were studied. An in vivo protective effect of GSTD on myocardial cells from neonatal rats was reported previously (Li et al., 2018). Likewise, Peng et al. have proven that cerebral ischemic damage in mice is alleviated by GSTD along with inhibition of cell apoptosis (Peng et al., 2015). A proliferation enhancing effects of GSTD has been reported by Zuo et al. in RSC96 Schwann cells (Zuo et al., 2016). In glutamate-treated PC12 cells, GSTD could reduce expression levels of Bax and cleaved caspase-3 (Jiang et al., 2014). Consistent with those previous studies, results in our study showed that GSTD pretreatment could attenuate hypoxic injury of H9c2 cells through promoting proliferation and repressing apoptosis, along with inhibition of the intrinsic apoptosis pathway.

The verified interaction between therapeutic drugs and miRNAs shed new light onto the mechanisms of potential drugs. We further studied the regulatory mechanism of GSTD focusing on the downstream miRNAs. miR-21, located on human chromosome 17 in the intronic region of the TMEM49 gene and highly expressed in adult heart, is an important miRNAs with pleiotropic functions (Cheng et al., 2007; Xu et al., 2014). It is proven to be a hypoxia-regulated miRNA and acts as a double-edged sword in ischemia/reperfusion injury (Xu et al., 2014). Up-regulation of miR-21 is observed in multiple cell types exposed to hypoxia, including neural stem/progenitor cells (Chen et al., 2017) and endothelial progenitor cells (Zuo et al., 2015). Moreover, miR-21, identified as an oncogene, exerts an anti-apoptotic function in ischemia/reperfusion- and hypoxia/reoxygenation-induced cardiomyocyte apoptosis via down-regulating Bax and inhibiting caspases (Yang et al., 2014). Yang et al. also demonstrated that trimetazidine could protect cardiomyocytes against hypoxia-reperfusion-induced apoptosis by increasing miR-21 expression (Yang et al., 2015). Combined those previous literatures and the results in our study which showed a protective role of GSTD in hypoxia-treated H9c2 cells, we hypothesized that there might be a correlation between GSTD and miR-21. Consistent with literatures described above, miR-21 was up-regulated in hypoxia-treated H9c2 cells. In addition, miR-21 level was further elevated by GSTD pretreatment in hypoxia-injured cells. Following experiments performed in transfected H9c2 cells verified that up-regulation of miR-21 was a possible explanation for the protective role of GSTD in hypoxia-injured H9c2 cells.

PDCD4 is confirmed as a novel tumor suppressor in various cancers, which also plays an important role in cardiomyocyte apoptosis (Jia et al., 2016). It has been reported that PDCD4 is an important functional target of the miR-21 in breast cancer cells (Frankel et al., 2008). Moreover, miR-21 has been proven to stimulate invasion, intravasation

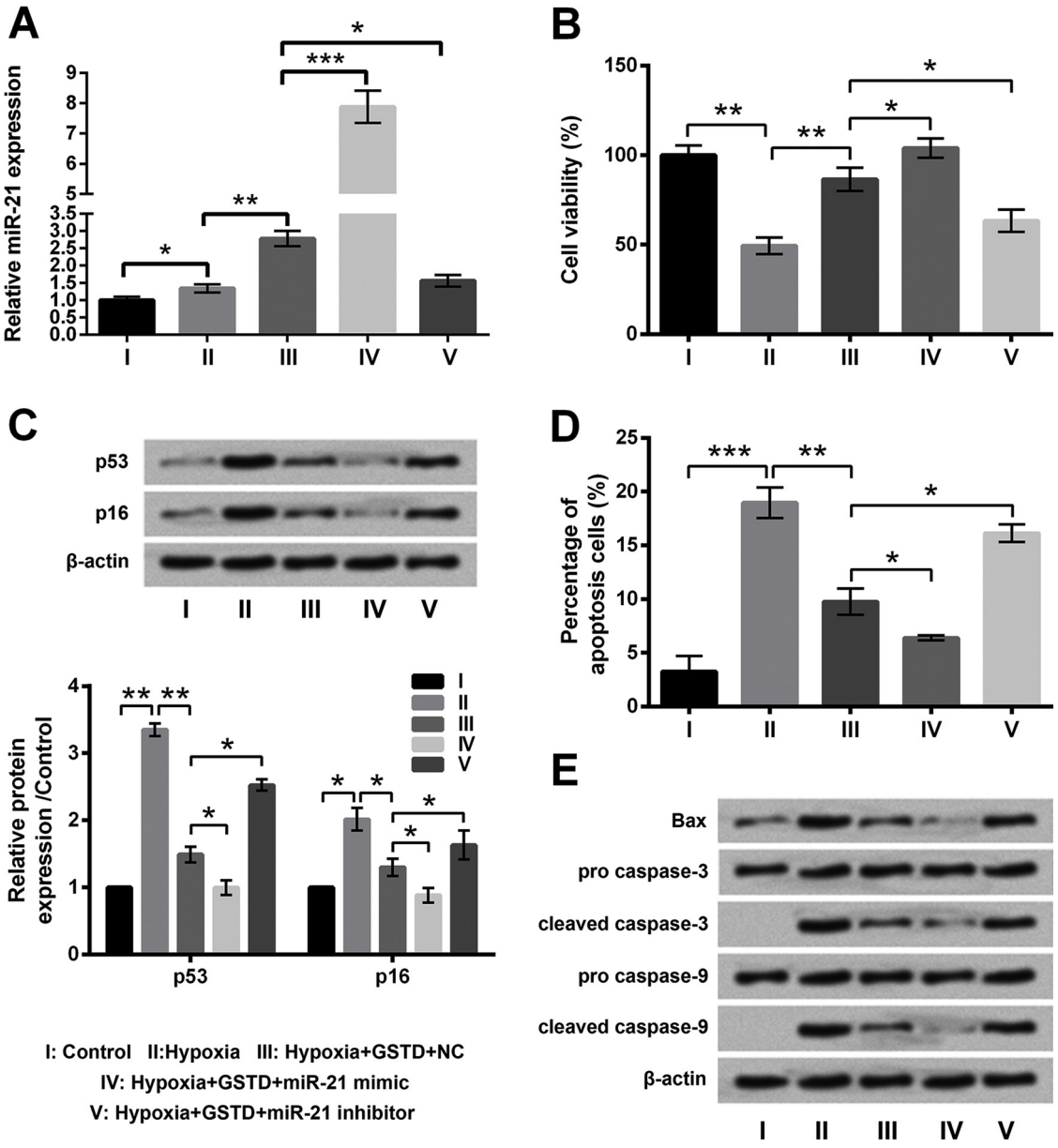

Fig. 5. Gastrodin (GSTD) affected hypoxia-treated H9c2 cells through up-regulating miR-21. H9c2 cells were transfected with miR-21 mimic, miR-21 inhibitor or their negative control (NC). Transfected H9c2 cells were stimulated with 10 μM GSTD for 2 h, followed by incubation under hypoxia for 12 h. (A) Expression of miR-21 was determined by stem-loop RT-PCR. Cells in the hypoxia group were incubated under hypoxia for 12 h. Non-treated cells were acted as control. (B) Cell viability was testified by CCK-8 assay. (C) Expression of p53 and p16 was evaluated by Western blot analysis. (D) Percentage of apoptotic cells was quantified by flow cytometry assay. (E) Expression of proteins associated with apoptosis was measured by Western blot analysis. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and metastasis in colorectal cancer by down-regulating PDCD4 (Asangani et al., 2008). In the present study, the bio-informatics and luciferase gene reporter assay revealed that PDCD4 was a direct target of miR-21 in H9c2 cells. More importantly, we found that PDCD4 obviously reversed the effect of miR-21 on hypoxia-injured H9c2 cells. These data indicated that PDCD4 might be a key regulator in MI, and the effect of GSTD on miR-21 expression might also be achieved by regulation of PDCD4. Further studies are still needed to confirm the conjecture.

The PI3K/AKT and NF-κB pathways are two signaling cascades that are crucial to diverse aspects of cell growth, survival and apoptosis (Porta et al., 2014; Taniguchi and Karin, 2018). Wang et al. have elucidated that hypoxia/reoxygenation injury of H9c2 cells is attenuated by shikonin through activation of the PI3K/AKT pathway (Wang et al., 2018). PI3K activation evokes production the phosphatidylinositol-3,4,5-triphosphate (PI3,4,5-P3), resulting in activation of AKT. PTEN is a negative regulator of PI3K/AKT due to the PI3,4,5-P3 is the main lipid substrate of PTEN (Porta et al., 2014). PTEN has been reported as a target gene of miR-21, which might be a possible reason for the negative correlation between miR-21 and PTEN expression (Liu et al., 2013). Recent study demonstrated that HNK inhibited the activation of PI3K/AKT signaling pathway through inhibiting miR-21 in human OS cells (Yang et al., 2018). Moreover, miR-21 regulated esophageal cancer cell proliferation, apoptosis, migration and invasion by targeting PTEN/PI3K/AKT signaling pathway (Wu et al., 2016). These studies indicated that miR-21 might be directly regulated PTEN/PI3K/AKT signaling pathway. Results in our study showed the PTEN/PI3K/AKT pathway was activated by GSTD via up-regulating miR-21. The effects of miR-21 on the PTEN/PI3K/AKT pathway were consistent with that on cardiac stem cells (Shi et al., 2017). The NF-κB pathway is a critical

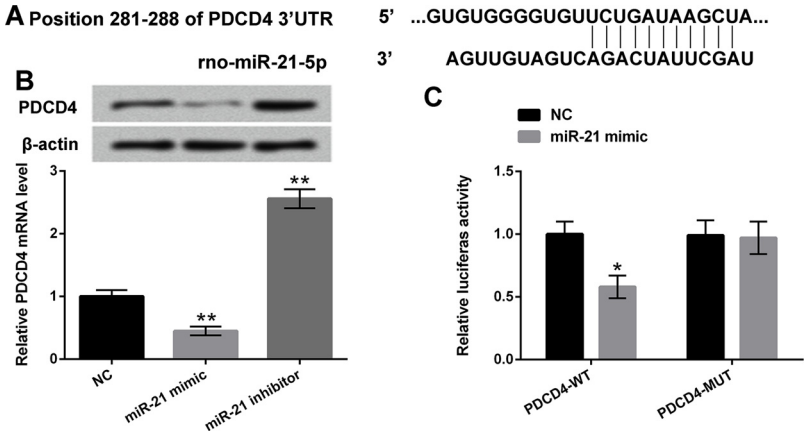

Fig. 6. Programmed cell death 4 (PDCD4) was a direct target of miR-21. (A) The location of the putative target site between PDCD4 and miR-21 were analyzed by the bioinformatics software of TargetScan (http://www.targetscan.org/) and microRNA database (http://www.microrna.org/). (B) The protein and mRNA levels of miR-21 were tested by Western blot and stem-loop RT-PCR assays. (C) The relationship between PDCD4 and miR-21 was examined by dual luciferase reporter assay. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

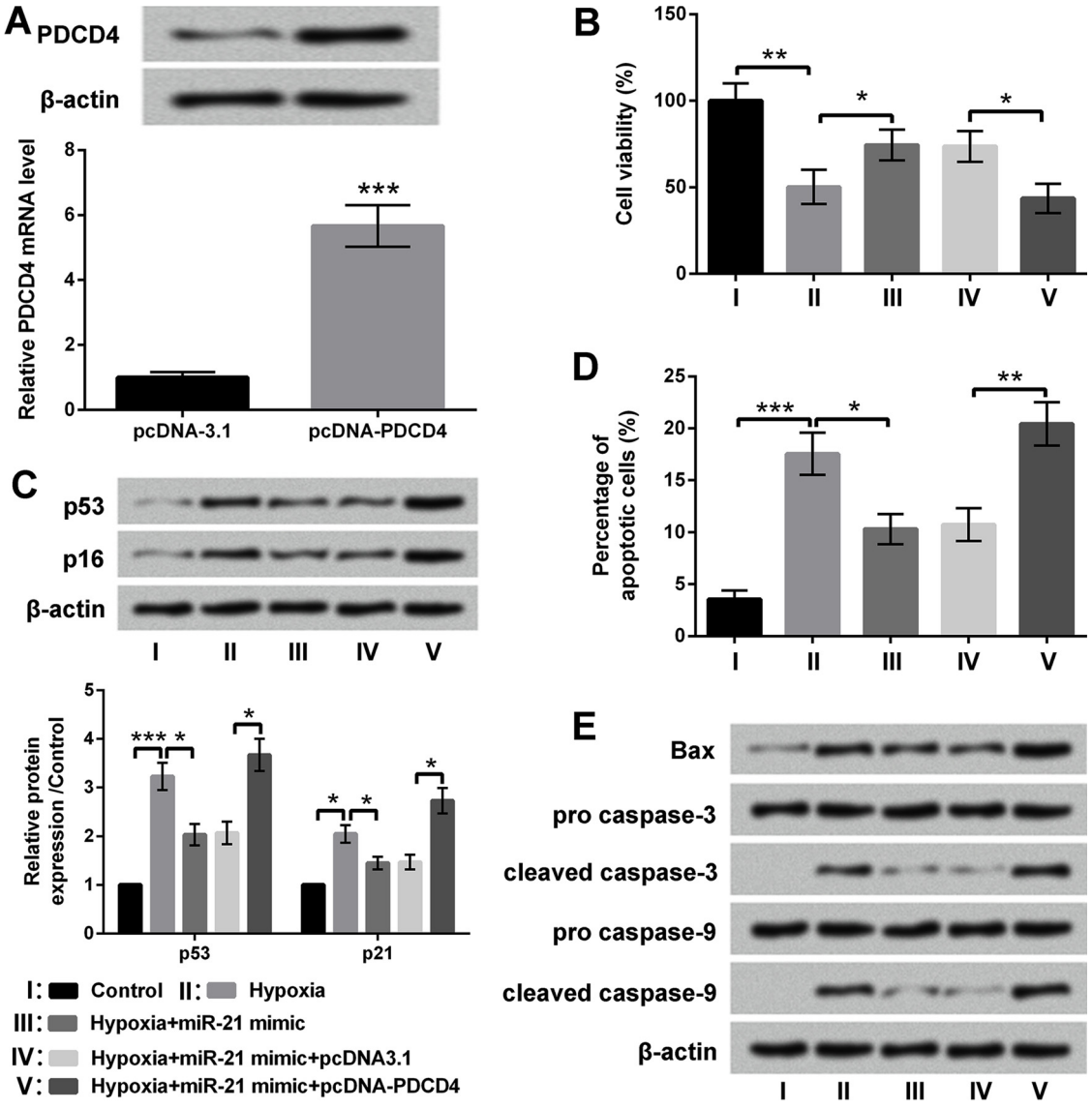

Fig. 7. Programmed cell death 4 (PDCD4) reversed the effect of miR-21 on hypoxia-treated H9c2 cells.
H9c2 cells were transfected with pcDNA-PDCD4 and pcDNA3.1. (A) Protein and mRNA levels of PDCD4 were determined by Western blot and stem-loop RT-PCR. The transfected cells were incubated under hypoxia for 12 h. Non-treated cells were acted as control. (B) Cell viability was examined by CCK-8 assay. (C) Expression of p53 and p16 was evaluated by Western blot analysis. (D) Percentage of apoptotic cells was determined by flow cytometry assay. (E) Expression of proteins associated with apoptosis was measured by Western blot analysis. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

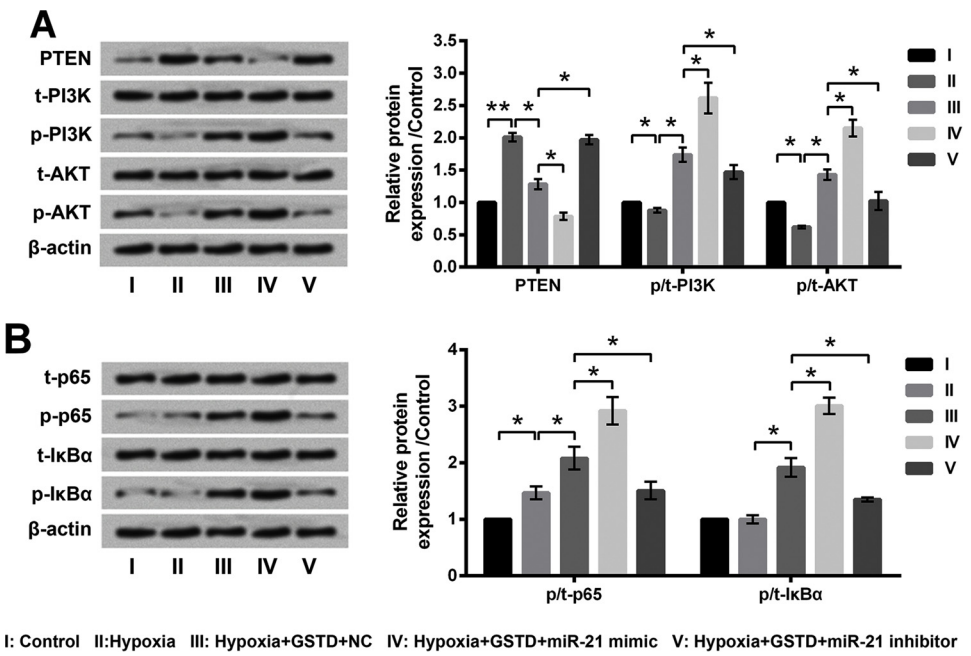

Fig. 8. Gastrodin (GSTD) activated the PTEN/PI3K/AKT and NF-κB pathways in hypoxia-treated H9c2 cells via regulation of miR-21. H9c2 cells transfected with miR-21 mimic, miR-21 inhibitor or their negative control (NC) were stimulated with 10 μM GSTD for 2 h, followed by incubation under hypoxia for 12 h. Cells in the hypoxia group were incubated under hypoxia for 12 h. Non-treated cells were acted as control. Expression of kinases in the PTEN/PI3K/AKT (A) and NF-κB (B) pathways was evaluated by Western blot analysis. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

downstream effector of AKT. Activated AKT may evoke phosphorylation of IκBα, and then give rise to release of p65 and translocation of p65 to the nucleus, resulting in activation of the NF-κB pathway (Li et al., 2016). Results in our study also proved that GSTD could activate the NF-κB pathway through up-regulation of miR-21.

In conclusion, the effects of GSTD on hypoxia-treated H9c2 cells were firstly studied. Results showed an *in vitro* protective effect of GSTD on cardiomyocytes. For study on the regulatory mechanism, we found that miR-21 was a downstream factor of GSTD, and GSTD might affect hypoxia-injured H9c2 cells through up-regulating miR-21. Additionally, PDCD4 was confirmed to be a direct target gene of miR-21, and PDCD4 overexpression reversed the effect of miR-21 on hypoxia-treated H9c2 cells. Besides, the PTEN/PI3K/AKT and NF-κB pathways were activated by GSTD via regulation of miR-21. This study provides data for the fundamental research of GSTD. The potential protective role of GSTD in hypoxia-injured cardiomyocytes makes GSTD become a candidate for the treatment of MI. However, the limitation of the study lies in the lack of the experiment to uncover the effect of PDCD4 on PTEN/PI3K/AKT and NF-κB pathways, meanwhile, more clinical experiments or animal experiments are still needed in the future to support the results in our study.

Conflicts of interest

All authors declare that they have no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Asangani, I.A., Rasheed, S.A., Nikolova, D.A., Leupold, J.H., Colburn, N.H., Post, S., et al., 2008. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27, 2128–2136.
Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.
Boon, R.A., Dimmeler, S., 2015. MicroRNAs in myocardial infarction. Nat. Rev. Cardiol. 12, 135–142.
Brady, C.A., Attardi, L.D., 2010. p53 at a glance. J. Cell. Sci. 123, 2527–2532.
Chang, W., Wu, Q.Q., Xiao, Y., Jiang, X.H., Yuan, Y., Zeng, X.F., et al., 2017. Acacetin protects against cardiac remodeling after myocardial infarction by mediating MAPK and PI3K/Akt signal pathway. J. Pharmacol. Sci. 135, 156–163.
Chen, R., Liu, Y., Su, Q., Yang, Y., Wang, L., Ma, S., et al., 2017. Hypoxia stimulates proliferation of rat neural stem/progenitor cells by regulating mir-21: an in vitro study. Neurosci. Lett. 661, 71–76.
Cheng, Y., Ji, R., Yue, J., Yang, J., Liu, X., Chen, H., et al., 2007. MicroRNAs are aberrantly expressed in hypertrophic heart: do they play a role in cardiac hypertrophy? Am. J. Pathol. 170, 1831–1840.
Chen-Scarabelli, C., Saravolatz Jr, L., Murad, Y., Shieh, W.S., Qureshi, W., Di Rezze, J., et al., 2012. A critical review of the use of carvedilol in ischemic heart disease. Am. J. Cardiovasc. Drugs 12, 391–401.
DeFilippis, A.P., Trainor, P.J., Hill, B.G., Amraotkar, A.R., Rai, S.N., Hirsch, G.A., et al., 2017. Identification of a plasma metabolomic signature of thrombotic myocardial infarction that is distinct from non-thrombotic myocardial infarction and stable coronary artery disease. PLoS One 12, e0175591.
Duan, L., Lei, H., Zhang, Y., Wan, B., Chang, J., Feng, Q., et al., 2016. Calcitonin gene-related peptide improves hypoxia-induced inflammation and apoptosis via nitric oxide in H9c2 cardiomyoblast cells. Cardiology 133, 44–53.
Feriani, D.J., Coelho-Júnior, H.J., de Oliveira, J.C.M.F., Delbin, M.A., Mostarda, C.T., Dourado, P.M.M., et al., 2018. Pyridostigmine improves the effects of resistance exercise training after myocardial infarction in rats. Front. Physiol. 9, 53.
Frankel, L.B., Christoffersen, N.R., Jacobsen, A., Lindow, M., Krogh, A., Lund, A.H., 2008. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J. Biol. Chem. 283, 1026–1033.
Fuhrmann, D.C., Brune, B., 2017. Mitochondrial composition and function under the control of hypoxia. Redox Biol. 12, 208–215.
Grans, C.F., Feriani, D.J., Abssamra, M.E., Rocha, L.Y., Carrozzi, N.M., Mostarda, C., et al., 2014. Resistance training after myocardial infarction in rats: its role on cardiac and autonomic function. Arq. Bras. Cardiol. 103, 60–68.
Gu, Y., Liang, Z., Wang, H., Jin, J., Zhang, S., Xue, S., et al., 2016. Tanshinone IIA protects H9c2 cells from oxidative stress-induced cell death via microRNA-133 upregulation and Akt activation. Exp. Ther. Med. 12, 1147–1152.
Gu, H., Liu, Z., Li, Y., Xie, Y., Yao, J., Zhu, Y., et al., 2018. Serum-derived extracellular vesicles protect against acute myocardial infarction by regulating miR-21/PDCD4 signaling pathway. Front. Physiol. 9, 348.
Jia, Z., Wang, J., Shi, Q., Liu, S., Wang, W., Tian, Y., et al., 2016. SOX6 and PDCD4 enhance cardiomyocyte apoptosis through LPS-induced miR-499 inhibition. Apoptosis 21, 174–183.
Jiang, G., Wu, H., Hu, Y., Li, J., Li, Q., 2014. Gastrodin inhibits glutamate-induced apoptosis of PC12 cells via inhibition of CaMKII/ASK-1/p38 MAPK/p53 signaling cascade. Cell. Mol. Neurobiol. 34, 591–602.
Keyes, K.T., Xu, J., Long, B., Zhang, C., Hu, Z., Ye, Y., 2010. Pharmacological inhibition of PTEN limits myocardial infarct size and improves left ventricular function post-infarction. Am. J. Physiol. Heart Circ. Physiol. 298, H1198–208.
Kruiswijk, F., Labuschagne, C.F., Vousden, K.H., 2015. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. Nat. Rev. Mol. Cell Biol. 16, 393–405.
Le, L.V., Mohindra, P., Fang, Q., Sievers, R.E., Mkrtschjan, M.A., Solis, C., et al., 2018. Injectable hyaluronic acid based microrods provide local micromechanical and biochemical cues to attenuate cardiac fibrosis after myocardial infarction. Biomaterials 169, 11–21.
Li, Z., Yang, Z., Passaniti, A., Lapidus, R.G., Liu, X., Cullen, K.J., et al., 2016. A positive feedback loop involving EGFR/Akt/mTORC1 and IKK/NF-κB regulates head and neck squamous cell carcinoma proliferation. Oncotarget 7, 31892–31906.

Li, X., Zhu, Q., Liu, Y., 2018. Gastrodin protects myocardial cells against hypoxia/reoxygenation injury in neonatal rats by inhibiting cell autophagy through the activation of mTOR signals in PI3K-Akt pathway. J Pharm Pharmacol. 70, 259–267.
Liu, Z.L., Wang, H., Liu, J., Wang, Z.X., 2013. MicroRNA-21 (miR-21) expression promotes growth, metastasis, and chemo- or radioresistance in non-small cell lung cancer cells by targeting PTEN. Mol. Cell. Biochem. 372, 35–45.
Liu, X., Deng, Y., Xu, Y., Jin, W., Li, H., 2018. MicroRNA-223 protects neonatal rat cardiomyocytes and H9c2 cells from hypoxia-induced apoptosis and excessive autophagy via the Akt/mTOR pathway by targeting PARP-1. J. Mol. Cell. Cardiol. 118, 133–146.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25, 402–408.
Mozaffarian, D., Benjamin, E.J., Go, A.S., Arnett, D.K., Blaha, M.J., Cushman, M., et al., 2015. Heart disease and stroke statistics–2015 update: a report from the American Heart Association. Circulation 131, e29–322.
Onai, Y., Suzuki, J., Maejima, Y., Haraguchi, G., Muto, S., Itai, A., et al., 2007. Inhibition of NF-{kappa}B improves left ventricular remodeling and cardiac dysfunction after myocardial infarction. Am. J. Physiol. Heart Circ. Physiol. 292, H530–538.
Peng, Z., Wang, S., Chen, G., Cai, M., Liu, R., Deng, J., et al., 2015. Gastrodin alleviates cerebral ischemic damage in mice by improving anti-oxidant and anti-inflammation activities and inhibiting apoptosis pathway. Neurochem. Res. 40, 661–673.
Porta, C., Paglino, C., Mosca, A., 2014. Targeting PI3K/Akt/mTOR signaling in Cancer. Front. Oncol. 4, 64.
Romagosa, C., Simonetti, S., Lopez-Vicente, L., Mazo, A., Lleonart, M.E., Castellvi, J., et al., 2011. p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene 30, 2087–2097.
Shi, B., Deng, W., Long, X., Zhao, R., Wang, Y., Chen, W., et al., 2017. miR-21 increases c-kit(+) cardiac stem cell proliferation in vitro through PTEN/PI3K/Akt signaling. PeerJ 5, e2859.
Song, L., Liu, S., Zhang, L., Yao, H., Gao, F., Xu, D., et al., 2016. MiR-21 modulates radiosensitivity of cervical cancer through inhibiting autophagy via the PTEN/Akt/HIF-1alpha feedback loop and the Akt-mTOR signaling pathway. Tumour Biol. 37, 12161–12168.
Taniguchi, K., Karin, M., 2018. NF-kappaB, inflammation, immunity and cancer: coming of age. Nat. Rev. Immunol. 18, 309–324.
Thornton, C., Hagberg, H., 2015. Role of mitochondria in apoptotic and necroptotic cell death in the developing brain. Clin. Chim. Acta 451, 35–38.
Wang, S., Zhu, Y., Qiu, R., 2018. Shikonin protects H9C2 cardiomyocytes against hypoxia/reoxygenation injury through activation of PI3K/Akt signaling pathway. Biomed. Pharmacother. 104, 712–717.
Wu, Y.R., Qi, H.J., Deng, D.F., Luo, Y.Y., Yang, S.L., 2016. MicroRNA-21 promotes cell proliferation, migration, and resistance to apoptosis through PTEN/PI3K/AKT signaling pathway in esophageal cancer. Tumour Biol. 37, 12061–12070.
Wu, J., Sun, C., Wang, R., Li, J., Zhou, M., Yan, M., et al., 2018. Cardioprotective effect of paeonol against epirubicin-induced heart injury via regulating miR-1 and PI3K/AKT pathway. Chem. Biol. Interact. 286, 17–25.
Xu, X., Kriegel, A.J., Jiao, X., Liu, H., Bai, X., Olson, J., et al., 2014. miR-21 in ischemia/reperfusion injury: a double-edged sword? Physiol. Genomics 46, 789–797.
Yang, P., Han, Y., Gui, L., Sun, J., Chen, Y.L., Song, R., et al., 2013. Gastrodin attenuation of the inflammatory response in H9c2 cardiomyocytes involves inhibition of NF-kappaB and MAPKs activation via the phosphatidylinositol 3-kinase signaling. Biochem. Pharmacol. 85, 1124–1133.
Yang, Q., Yang, K., Li, A., 2014. microRNA-21 protects against ischemia-reperfusion and hypoxia-reperfusion-induced cardiocyte apoptosis via the phosphatase and tensin homolog/Akt-dependent mechanism. Mol. Med. Rep. 9, 2213–2220.
Yang, Q., Yang, K., Li, A.-Y., 2015. Trimetazidine protects against hypoxia-reperfusion-induced cardiomyocyte apoptosis by increasing microRNA-21 expression. Int. J. Clin. Exp. Pathol. 8, 3735–3741.
Yang, J., Zou, Y., Jiang, D., 2018. Honokiol suppresses proliferation and induces apoptosis via regulation of the miR21/PTEN/PI3K/AKT signaling pathway in human osteosarcoma cells. Int. J. Mol. Med. 41, 1845–1854.
Yuan, J., Chen, H., Ge, D., Xu, Y., Xu, H., Yang, Y., et al., 2017. Mir-21 promotes cardiac fibrosis after myocardial infarction via targeting Smad7. Cell. Physiol. Biochem. 42, 2207–2219.
Zhang, Z., Li, Z., Gao, C., Chen, P., Chen, J., Liu, W., et al., 2008. miR-21 plays a pivotal role in gastric cancer pathogenesis and progression. Lab. Invest. 88, 1358–1366.
Zhang, J.S., Zhou, S.F., Wang, Q., Guo, J.N., Liang, H.M., Deng, J.B., et al., 2016. Gastrodin suppresses BACE1 expression under oxidative stress condition via inhibition of the PKR/eIF2alpha pathway in Alzheimer's disease. Neuroscience 325, 1–9.
Zuo, K., Li, M., Zhang, X., Lu, C., Wang, S., Zhi, K., et al., 2015. MiR-21 suppresses endothelial progenitor cell proliferation by activating the TGFβ signaling pathway via downregulation of WWP1. Int. J. Clin. Exp. Pathol. 8, 414–422.
Zuo, W., Xu, F., Zhang, K., Zheng, L., Zhao, J., 2016. Proliferation-enhancing effects of gastrodin on RSC96 Schwann cells by regulating ERK1/2 and PI3K signaling pathways. Biomed. Pharmacother. 84, 747–753.