Original Paper

Ganoderic Acid A Protects Rat H9c2 Cardiomyocytes from Hypoxia-Induced Injury via Up-Regulating miR-182-5p

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Key Words

Ganoderic acid A • Hypoxia • H9c2 cardiomyocytes • microRNA-182-5p • PTEN • PI3K/AKT signaling pathway

Abstract

Background/Aims: Ganoderic acid A (GAA) isolated from *Ganoderma lucidum*, shows various benefit activities, such as anti-tumor activity, anti-HIV activity and hepatoprotective activity. However, the potential effect of GAA on hypoxia-induced injury of cardiomyocytes are still unclear. In this study, we aimed to reveal the effects of GAA on hypoxia-induced H9c2 cell injury, as well as potential underlying mechanisms. **Methods:** Rat H9c2 cardiomyocytes were cultured in hypoxia condition with different doses of GAA. Cell viability and apoptosis were detected by CCK-8 assay and flow cytometry, respectively. qRT-PCR was performed to assess the expression level of microRNA-182-5p (miR-182-5p) and phosphatase and tensin homologue (PTEN). Transfection was conducted to change the expression levels of miR-182-5p and PTEN in H9c2 cells. Finally, protein levels of key factors involved in cell proliferation, cell apoptosis and PI3K/AKT pathway were evaluated using western blotting. **Results:** Hypoxia treatment significantly induced H9c2 cell viability loss and apoptosis. GAA incubation remarkably protected H9c2 cells from hypoxia-induced viability loss, proliferation inhibition and apoptosis. In addition, GAA obviously enhanced the expression level of miR-182-5p in H9c2 cells. Suppression of miR-182-5p notably alleviated the protective effects of GAA on hypoxia-treated H9c2 cells. Furthermore, miR-182-5p negatively regulated the mRNA and protein levels of PTEN in H9c2 cells. GAA attenuated hypoxia-induced inactivation of PI3K/AKT pathway in H9c2 cells by up-regulating miR-182-5p and then down-regulating PTEN. **Conclusion:** GAA protected rat H9c2 cardiomyocytes from hypoxia-induced injury might via up-regulating miR-182-5p, down-regulating PTEN and then activating PI3K/AKT signaling pathway.

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Introduction

Coronary artery diseases (CAD), also known as ischemic heart diseases, is a group of diseases that includes stable angina, unstable angina, myocardial infarction (MI), and sudden cardiac death [1]. Despite the fact that the diagnose and treatment of CAD have been largely improved in the last several decades, there are also still lots of people died of CAD globally [2]. MI is occurred by acute, persistent ischemic or hypoxia of heart, and causes injury of myocardial cells [3]. Significant apoptosis of cardiomyocytes is an important pathogenesis feature of MI [4]. Hypoxia is able to damage cardiomyocytes and trigger cardiomyocytes apoptosis in the process of acute and chronic MI [3, 5]. It is worthy believing that the search for novel and more effective therapeutic medicines that can protect cardiomyocytes from hypoxia-induced injury will be helpful for the treatment of MI.

Ganoderma lucidum (*G. lucidum*) has been used as a traditional medicine with few side effects in East Asia for thousands of years [6]. Pharmacological researches have demonstrated that *G. lucidum* has a wide range of benefic effects to treat multiple diseases, including angina pectoris, hypercholesterolemia, atherosclerosis, hypertension, hepatic diseases, diabetes, cancer and bronchitis [7–13]. Recent years, *G. lucidum* is becoming increasingly popular in western countries as a complementary medicine in cardiovascular disease therapy [11, 14]. More than 140 triterpenoids have been isolated from *G. lucidum* and we believe that they have a variety of pharmacological activities [15]. Ganoderic acid, one of the most important classes of these triterpenoids, among which ganoderic acid A (GAA) has been demonstrated to exhibit anti-tumor activity [16], anti-HIV activity [17], inhibitory activity against protein prenyltransferase [18], beta-glucuronidase-inhibitory activity [19], hepato-protective activity [19] and anti-nociceptive activity [20]. However, there is little information available about the effects of GAA on hypoxia-induced cardiomyocytes injury.

MicroRNAs (miRNAs), a class of single-stranded endogenous, non-coding and small (~22 nucleotides in length) RNAs, have been widely reported to regulate multiple gene expression in cells [21]. Moreover, abnormal expression of specific miRNAs have been found to participate in the pathogenesis of many heart diseases [22]. Numerous medicines can exert benefic activities in heart diseases by regulating the expression of miRNAs [23]. miRNA-182-5p (miR-182-5p) has been found to protect H9c2 cardiomyocytes from hypoxia-induced apoptosis [24].

Hence, in our present study, rat H9c2 cardiomyocytes were cultured in hypoxia condition to stimulate MI cell injury. Then, the effects of GAA on hypoxia-induced H9c2 cell viability loss, proliferation inhibition and apoptosis were analyzed. More importantly, the possible interrelationship mechanism involving in miR-182-5p was investigated. Our study indicates that GAA protects H9c2 cells from hypoxia-induced injury might through up-regulating miR-182-5p expression, and thereby modulating phosphatase and tensin homolog (PTEN)/PI3K/AKT signaling pathway.

Materials and Methods

Cell culture and treatment

H9c2 cells, derived from rat embryonic ventricular cardiomyocytes, were purchased from the American Type Culture Collection (Catalogue No.CRL-1446™; ATCC, Manassas, VA, USA). H9c2 cells were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen, San Diego, CA) with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 IU/ml penicillin G (Gibco) and 100 µg/ml streptomycin (Gibco) at 37°C in humidity incubator (Sanyo, Jencons, UK) with 5% CO₂.

To establish hypoxia-induced cardiomyocytes injury model, H9c2 cells were subjected to hypoxic (1% O₂, 5% CO₂ balanced with N₂) culture for 12 h. Cells cultured in normoxia (room air, 5% CO₂) were as control.

GAA (purity ≥ 98%) was purchased from Sigma-Aldrich (SMB00445; St Louis, MO, USA). A stock of GAA solution with a concentration of 10 g/L was prepared by dissolving it in methanol (Sigma-Aldrich) and

stored at 4°C. GAA with different concentrations (2, 20 and 100 µg/ml) was used to treat cells throughout the hypoxia exposure. The H9c2 cells treated with 0.2 ml 1% methanol were used as a control.

Cell viability assay

H9c2 cells (5000 cells/well) were seeded into 96-well plates with 1×10^4 cells per well. Cell viability was assessed by Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA) assay. Briefly, after hypoxic treatment for 12 h with or without different doses of GAA (0, 2, 20 and 100 µg/ml) incubation or miR-182-5p inhibitor transfection, CCK-8 solution (10 µl) was added into the culture mediums of each well of the plate and the plates were incubated for 1 h at 37°C with 5% CO₂. After that, the absorbance of each well was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA). Cell viability (%) was calculated by average absorbance of treatment group/average absorbance of control group $\times 100\%$.

Cell apoptosis assay

Cell apoptosis assay was performed by flow cytometry using Annexin V-FITC apoptosis detection kit (Invitrogen, Camarillo, CA, USA) according to the manufacturer's instruction. H9c2 cells (1×10^5 cells/well) were seeded into 6 well-plates. After hypoxic treatment for 12 h with or without different doses of GAA (0, 2, 20 and 100 µg/ml) incubation or miR-182-5p inhibitor transfection, cells were collected and washed twice with cold phosphate buffered saline (PBS) and re-suspended in 100 µl binding buffer. Then, cells were stained using 5 µl of Annexin V-FITC and 1 µl of PI. After incubation in the dark at room temperature for 15 minutes, the stained cells were analyzed with flow cytometer (BD FACSscan Coulter, Fullerton, CA, USA) at 530 nm emission to differentiate apoptotic cells (Annexin V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive). Data was analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Cell transfection

miR-182-5p inhibitor, miR-182-5p mimic and negative control (NC) were synthesized by GenePharma Corporation (Shanghai, China). The sequence of PTEN was constructed into pEX vector (GenePharma) and referred as pEX-PTEN. The empty vector was used as NC of pEX-PTEN and referred as pEX. Cell transfection was conducted using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The stably transfected cells were selected by the culture medium containing 0.5 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA).

Real-time quantitative reverse-transcriptase PCR (qRT-PCR)

After relevant treatment, total RNAs was extracted from H9c2 cells using Trizol Reagent (Invitrogen). For quantifying the expression of miR-182-5p, cDNA was synthesized using universal cDNA synthesis kit II (Exiqon, Copenhagen, Denmark) and qPCR was performed using ExiLERATE SYBR® Green Master Mix II (Exiqon) according to manufacturer's protocols. U6 (Exiqon) was used for normalizing the expression of miR-182-5p. For analyzing the mRNA expression of PTEN, PrimeScript RT reagent Kit (TaKaRa, Dalian, China) and SYBR Premix ExTaq Kit (TaKaRa) were used in turns according to manufacturer's protocols for reverse transcription and qPCR. β -actin (TaKaRa) was used as an internal control. The relative expression rate was calculated by the $2^{-\Delta\Delta CT}$ method.

Western blotting

The total proteins for western blotting were extracted using RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) and quantified using the BCA Protein Assay Kit (Pierce, Appleton, WI, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein bands. Then, protein bands were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) followed with blocking with 5% skim milk (Nestlé, Shuangcheng, China) in Tris Buffered Saline with Tween (TBST) for 2 h. Then, the membranes were incubated with corresponding primary antibodies at 4°C overnight and incubated with secondary antibodies marked by horseradish peroxidase for 1 h at room temperature. All antibodies were purchased from Abcam Biotechnology (Cambridge, UK). The primary antibodies against PCNA (ab29, 1:1000), Cyclin A2 (ab38, 1:200), Cyclin E1 (ab71535, 1:2000), CDK2 (ab32147, 1:5000), CDK4 (ab199728, 1:2000), Cyclin D1 (ab134175, 1:20000), Bcl-2 (ab59348, 1:1000), Bax (ab182733, 1:2000), pro-caspase-3 (ab44976, 1:500), cleaved-caspase-3 (ab13847, 1:500),

cytochrome c (ab13575, 1:1000), PTEN (ab32199, 1:10000), PI3K (ab151549, 1:1000), p-PI3K (ab182651, 1:1000), AKT (ab179463, 1:10000), p-AKT (ab38449, 1:1000), and β -actin (ab8224, 1:1000) were used. β -actin antibody was used as the endogenous protein for reference. Secondary antibodies included goat anti-mouse IgG (ab6789, 1:5000) and goat anti-rabbit IgG (ab6721, 1:5000). Blots were visualized using the ECL kit (Amersham Pharmacia Biotech, Rainham, UK).

Statistical analysis

All experiments were repeated three times. Data was presented as the mean $\pm$ standard deviation (SD). SPSS 19.0 statistical software was used for statistical analyses. The *P*-values were calculated using a one-way analysis of variance (ANOVA). A *P*-value of < 0.05 was considered to be statistically significant.

Results

Hypoxia inhibited H9c2 cell viability and induced cell apoptosis

After culturing in hypoxic or control (normoxic) environment for 12 h, viability and apoptosis of H9c2 cells were analyzed. As shown in Fig. 1A, CCK-8 assay showed that viability of H9c2 cells was significantly decreased after 12 h of hypoxia incubation ($P < 0.01$). Flow cytometry analysis demonstrated that the rate of apoptotic cells was significantly increased after hypoxic treatment (Fig. 1B; $P < 0.01$). These results indicated that 12 h of hypoxic incubation significantly reduced H9c2 cell viability and induced cell apoptosis. Therefore, this hypoxia-induced cardiomyocytes injury model was used for subsequent experiments.

GAA protected H9c2 cells from hypoxia-induced viability loss, proliferation inhibition and apoptosis

To reveal the potential protective effects of GAA on hypoxic-induced H9c2 cell injury, H9c2 cells were treated with different doses of GAA (2, 20 and 100 μ g/ml) under hypoxic condition for 12 h. Then, the viability, proliferation and apoptosis of treated cells were analyzed. As shown in Fig. 2, compared with hypoxia single incubation, GAA treatment significantly increased the viability of H9c2 cells at concentrations of 20 and 100 μ g/ml ($P < 0.01$). The protein expression levels of proliferation-related factors in H9c2 cells after hypoxia incubation and 100 μ g/ml GAA treatment were detected by western blotting. Fig. 2B showed that hypoxia incubation reduced the protein expression levels of PCNA, Cyclin E1, CDK2, Cyclin D1 and CDK4 in H9c2 cells. GAA (100 μ g/ml) treatment reversed the hypoxia incubation-induced expression decreases of PCNA, Cyclin E1, CDK2, Cyclin D1 and CDK4 in H9c2 cells. Cyclin A2 expression did not change in H9c2 cells under hypoxia with or without GAA treatment. These results suggested that GAA could alleviate hypoxia-induced proliferation inhibition of H9c2 cells. The results of Flow cytometry analysis demonstrated that compared to hypoxia single incubation, the apoptotic rates of H9c2 cells were significantly decreased after GAA treatment at concentrations of 20 and 100 μ g/ml (Fig.

1. Hypoxia inhibited H9c2 cell viability and induced cell apoptosis. The viability of H9c2 cell viability was analyzed by CCK-8 assay after hypoxic treatment for 12 h (B). The apoptosis of H9c2 cells was detected by flow cytometry analysis after hypoxic treatment for 12 h. ** $P < 0.01$.

2C; $P < 0.01$). The protein expression levels of apoptosis-related factors were also detected by western blotting. Fig. 2D showed that GAA administration (100 $\mu\text{g/ml}$) resulted in an increase of anti-apoptotic protein Bcl-2 expression and decreases of pro-apoptotic proteins Bax, cleaved-caspase-3 and cytochrome c in H9c2 cells. These results suggested that GAA could protect H9c2 cells from hypoxic-induced viability loss, proliferation inhibition and apoptosis.

GAA up-regulated miR-182-5p expression in H9c2 cells

Next, we want to identify the effect of GAA on miR-182-5p expression in H9c2 cells. The expression of miR-182-5p in H9c2 cells with GAA administration (100 $\mu\text{g/ml}$) under hypoxia condition was detected by qRT-PCR. Fig. 3 showed that GAA (100 $\mu\text{g/ml}$) treatment remarkably increased the expression of miR-182-5p in H9c2 cells ($P < 0.05$). This result

Fig. 2. GAA protected H9c2 cells from hypoxia-induced viability loss, proliferation inhibition and apoptosis. (A) H9c2 cells were treated with different doses of GAA (0, 2, 20, 100 $\mu\text{g/ml}$) under hypoxic culture for 12 h. Then, the viability of cells was detected by CCK-8 assay. (B) Expression levels of proliferation-related factors in H9c2 cells were detected by western blotting after treated with 100 $\mu\text{g/ml}$ of GAA under hypoxia condition. (C) Apoptosis of H9c2 cells was detected by flow cytometry after treated with different doses of GAA (0, 2, 20, 100 $\mu\text{g/ml}$) under hypoxia condition. (D) Expression levels of apoptosis-related factors in H9c2 were detected by western blotting after treated with 100 $\mu\text{g/ml}$ of GAA under hypoxia condition. ns, no significant, $**P < 0.01$.

suggested that GAA positively regulated the expression of miR-182-5p in H9c2 cells and implied that miR-182-5p might be involved in the protective effects of GAA on hypoxia-induced H9c2 cell injury.

GAA protected H9c2 cells against hypoxia-induced injury by up-regulating miR-182-5p expression

We further investigated the protective effects of GAA on the hypoxia-induced H9c2 cell viability loss, proliferation inhibition and apoptosis in the case of miR-182-5p silence. Fig. 4A showed that transfection with miR-182-5p inhibitor effectively reduced the expression of miR-182-5p in H9c2 cells ($P < 0.01$). Therefore, H9c2 cells transfected with miR-182-5p inhibitor were used for the subsequent experiments. These transfected cells were cultured in hypoxia condition with or without GAA (100 $\mu\text{g}/\text{ml}$, 2 h) treatment, followed by analyzing cell proliferation and apoptosis. As shown in Fig. 4B, compared to hypoxia+GAA+NC group, the viability of H9c2 cells was significantly decreased in hypoxia+GAA+miR-182-5p inhibitor group ($P < 0.01$), which suggested that the expression of miR-182-5p reversed the protective effects of GAA on hypoxic-induced H9c2 cell viability loss. Moreover, similar results were found in Fig. 4C, which showed that compared to hypoxia+GAA+NC group, the protein expression levels of PCNA, Cyclin D1, CDK2, Cyclin D1 and CDK4 were all decreased in hypoxic+GAA+ miR-182-5p inhibitor group.

In addition, the results in Fig. 4D showed that the rate of apoptotic cells was notably increased in hypoxia+GAA+miR-182-5p inhibitor group, compared to hypoxic+GAA+NC group ($P < 0.01$). Moreover, Western blotting showed that compared to hypoxia+GAA+NC group, the protein expression level of Bcl-2 was reduced, as well as the protein expression levels of Bax, cleaved-caspase-3 and cytochrome c were enhanced in hypoxia+GAA+miR-182-5p inhibitor group. Considering that GAA positively regulated miR-182-5p expression, we then concluded that GAA protected H9c2 cells from hypoxia-induced viability loss, proliferation inhibition and apoptosis might via up-regulating miR-182-5p expression.

miR-182-5p negatively regulated the expression of PTEN in H9c2 cells

Previous study reported that inhibition of PTEN could promote cell survival in heart [26, 27]. We next identified the regulatory effect of miR-182-5p on PTEN expression in both mRNA and protein levels. As shown in Fig. 5A, the mRNA expression level of PTEN was decreased after overexpression of miR-182-5p, while it was increased after miR-182-5p silencing ($P < 0.01$). Western blotting showed that overexpression of miR-182-5p caused a decrease of PTEN protein expression, while miR-182-5p silence had an inverse effect (Fig. 5B). These results indicated that miR-182-5p negatively regulated PTEN expression in both mRNA and protein levels in H9c2 cells.

GAA activated PI3K/AKT signaling pathway in H9c2 cells by up-regulating miR-182-5p and down-regulating PTEN

Given that, PI3K/AKT signaling pathway plays protective roles in the survival of cardiomyocytes [27]. Moreover, PTEN is a negative regulator of PI3K/AKT pathway [28]. We evaluated the effects of hypoxia, GAA and miR-182-5p on expression levels of PTEN, PI3K and AKT in H9c2 cells. As shown in Fig. 5C, 100 $\mu\text{g}/\text{ml}$ GAA treatment causes a decrease of PTEN

Fig. 3. GAA up-regulated the expression of miR-182-5p in H9c2 cells. Expression level of miR-182-5p in H9c2 cells was measured by qPCR after treated with GAA administration (100 $\mu\text{g}/\text{ml}$, 2 h) under hypoxia condition. * $P < 0.05$.

Fig. 4. GAA protected H9c2 cells from hypoxia-induced injury by up-regulating miR-182-5p expression. (A) miR-182-5p expression in H9c2 cells was measured by qRT-PCR after transfection with miR-182-5p inhibitor. (B) Viability of H9c2 cells after GAA treatment and/or miR-182-5p inhibitor transfection under hypoxia condition for 12 h was detected by CCK-8 assay. (C) Expression levels of proliferation-related factors in H9c2 cells were detected by western blotting after GAA treatment and/or miR-182-5p inhibitor transfection under hypoxia condition for 12 h. (D) Apoptosis of H9c2 cells were detected by flow cytometry after GAA treatment and/or miR-182-5p inhibitor transfection under hypoxia condition for 12 h. (E) Expression levels of apoptosis-related factors in H9c2 cells were detected by western blotting after GAA treatment and/or miR-182-5p inhibitor transfection under hypoxia condition for 12 h. ** $P < 0.01$.

expression, but increases the phosphorylation levels of PI3K and AKT in H9c2 cells. These results indicated that GAA down-regulated PTEN expression and thereby contributed to the activation of PI3K/AKT signaling pathway in H9c2 cells under hypoxia condition. However, compared to hypoxia+GAA+NC group, the expression of PTEN was increased, as well as the phosphorylation levels of PI3K and AKT were decreased in hypoxia+GAA+miR-182-5p inhibitor group. Considering that GAA positively regulated miR-182-5p expression, these results indicated that GAA down-regulated PTEN expression and then activated PI3K/AKT signaling pathway under hypoxia condition possibly through up-regulating miR-182-5p expression. In addition, Fig. 5C also showed that PTEN overexpression dramatically decreased the phosphorylation levels of PI3K and AKT in H9c2 cells under hypoxia condition when GAA treatment was applied. These above results suggested that GAA activated PI3K/AKT signaling pathway in H9c2 cells at least in part by up-regulating miR-182-5p and down-regulating PTEN expression.

Discussion

The present study was aimed to investigate the protective effects of GAA on hypoxia-induced injury of cardiomyocytes, as well as potential underlying molecular mechanisms. We confirmed that hypoxia incubation significantly inhibited rat H9c2 cardiomyocytes viability and induced cell apoptosis. More importantly, we revealed that GAA remarkably protected H9c2 cells from hypoxia-induced viability and proliferation inhibition, as well as apoptosis, by up-regulating the expression of miR-182-5p. In addition, we found that GAA inhibited PTEN expression and thereby activated PI3K/AKT signaling pathway in H9c2 cells by up-regulating the expression of miR-182-5p.

GAA, a ganoderic acid isolated from *G. lucidum*, has been reported to exhibit various beneficial activities, such as anti-tumor activity, anti-HIV activity and hepato-protective activity [16-20]. However, there are only several literatures that reported the effects of GAA or *G. lucidum* on hypoxia-induced cell injury. A recent study demonstrated that triterpenes of *G. lucidum* could protect cerebral cortical neurons against hypoxia/reoxygenation injury [29]. Another report showed that *G. lucidum* extract prevented cultured human tubular epithelial cells from oxidative damage [30]. Moreover, GAA has been reported to show anti-oxidative effects against erythrocyte membrane oxidation and lipid peroxidation [31]. However, the

Fig. 5. GAA activates PI3K/AKT pathway in H9c2 cells by up-regulating miR-182-5p and down-regulating PTEN. mRNA and protein expression levels of PTEN in H9c2 cells were determined by (A) qRT-PCR and (B) Western blotting after transfection with miR-182-5p mimic or miR-182-5p inhibitor. (C) Protein expression levels of PTEN, PI3K and AKT in H9c2 cells were detected by Western blotting after relevant treatment or transfection under hypoxia condition. **P<0.01.

effects of GAA on hypoxic-induced cardiomyocytes injury remain unclear yet. In this study, we found that GAA effectively protected H9c2 cells from hypoxia-induced injury by increasing cell viability and proliferation, as well as reducing cell apoptosis, which suggested that GAA might exert protective effects on hypoxia-induced MI cell model.

Next, in order to reveal the potential underlying molecular mechanism of GAA protective effects on hypoxia-induced H9c2 cell injury, we investigated the association between GAA and miR-182-5p in hypoxic-treated H9c2 cells. miR-182-5p is a member of miR-182 family and can act as an oncogene in several types of cancers [32, 33]. Only a few previous studies have investigated the functions of miR-182 in the heart, which indicated that miR-182 was related to cardiac hypertrophy [34, 35]. In addition, a previous study showed that miR-182-5p could protect H9c2 cells from hypoxia-induced apoptosis [24]. In consistent with the report, in this study, we found that GAA up-regulated the expression of miR-182-5p in H9c2 cells under hypoxia condition. Suppression of miR-182-5p notably reversed the protective effects of GAA on hypoxic-induced H9c2 cell injury. These findings suggest that miR-182-5p may play a key role in the protective effects of GAA on hypoxia-treated H9c2 cells, although the cellular mechanism is unknown. Previous study reported that the regulation of miRNAs expression is very complex, which can be divided into transcriptional regulation and post-transcriptional regulation, and might be involved in multiple intracellular processes [23]. Thus, we propose that hypoxia and GAA regulated the expression of miR-182-5p at transcriptional level or post-transcriptional level. Further experimental research is still needed to analyze the underlying regulatory mechanism of hypoxia and GAA on miR-182-5p expression.

In the next step, we investigated the association between GAA, miR-182-5p, PTEN and PI3K/AKT signaling pathway in H9c2 cells under hypoxia condition. Activation of the PI3K/AKT pathway has been reported to protect heart from ischemia-reperfusion injury [36, 37] and prolong the survival of cardiomyocytes [37, 38]. Reports also showed that PTEN overexpression could increase the apoptosis of neonatal cardiomyocytes, while the inhibition of PTEN could activate the PI3K/AKT survival pathway, and reduce apoptosis [39, 40]. Thus, knockdown of PTEN has been demonstrated to be a beneficial way to protecting heart [39]. The findings of this study indicated that miR-182-5p negatively regulated the expression of PTEN in H9c2 cells. From TargetScan and microRNA database, we did not find any valuable information on miR-182-5p and PTEN. Thus, we propose that miR-182-5p influence the expression of PTEN in H9c2 cells might be via mediation of other factors. Which factor might be the direct target gene of miR-182-5p and interacted with the expression of PTEN still needs further investigations. Previous study reported that miR-182 could activate Akt by inhibiting PTEN activity to promote axon outgrowth [41]. Another study showed that miR-182-5p down-regulated PTEN expression to protect H9c2 cells from hypoxia-induced apoptosis [24]. These results suggested that GAA exerted protective effects on hypoxia-induced H9c2 cell injury at least in part by up-regulating miR-182-5p and then down-regulating PTEN. Moreover, we revealed that GAA decreased PTEN expression and then activated PI3K/AKT signaling pathway in H9c2 cells under hypoxia condition, which might contribute to the protective effects of GAA on hypoxic-induced H9c2 cell injury.

Conclusion

In conclusion, the present study revealed that GAA significantly protected rat H9c2 cardiomyocytes from hypoxia-induced injury might through up-regulating miR-182-5p expression, down-regulating PTEN and then activating PI3K/AKT pathways. Hence, our findings will be helpful for further understanding the protective effects of GAA on MI and provide theoretical basis for deeply exploring the treatment of MI by using GAA.

Disclosure Statement

The authors declare no conflicts of interest.

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