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Original Research Article

Pogostone alleviates angiotensin II-induced cardiomyocyte hypertrophy in H9c2 cells through MAPK and Nrf2 pathway

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Abstract

Purpose: *To investigate the effect of pogostone on cardiac hypertrophy.*
Methods: *An in vitro model of myocardial hypertrophy was first established by stimulating H9c2 (rat cardiomyocytes) with angiotensin II (Ang II), and the cells treated with or without pogostone. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were measured by western blot. Immunofluorescence staining was performed for α-actinin while cell surface area was quantified. Dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe and malondialdehyde (MDA) assay kit were used to determine reactive oxygen species (ROS) and MDA levels respectively. Apoptosis was evaluated by flow cytometry while Nrf2, p38, ERK, and JNK protein expression levels were determined by western-blot assay.*
Results: *Compared with the control group, ANP and BNP protein expression levels, cell surface area, ROS, MDA, and apoptosis were all elevated in H9c2 cells after stimulation with Ang II ($p < 0.001$). Varying doses of pogostone decreased protein expressions of ANP and BNP, reduced cell surface area, decreased ROS and MDA levels, and inhibited apoptosis. Pogostone also up-regulated and inhibited the phosphorylation levels of p38 and ERK, and JNK levels in H9c2 cells.*
Conclusion: *Pogostone reduces protein expression of ANP and BNP and up-regulated Nrf2 protein expression in H9c2 cells stimulated with angiotensin II.*

Keywords: *Pogostone, Cardiomyocyte Hypertrophy, Nuclear Factor erythroid 2-Related Factor 2, Mitogen-activated protein kinases, Reactive oxygen species*

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INTRODUCTION

Myocardial hypertrophy is characterized by increased myocardial cell size, cell death, and fibrosis. It mediates cardiac enlargement and is a compensatory response. With continued stimulation, myocardial hypertrophy transforms into pathologically hypertrophic remodeling and dysfunction of the heart, which ultimately results in heart failure and increased mortality. Therefore, finding effective potential drugs is crucial for the treatment of cardiac hypertrophy [1,2].

Angiotensin II (Ang II) is essential for controlling vascular structure and function, and by inducing

oxidative stress, it contributes to cardiovascular disease progression. Ang II binds to angiotensin II type 1 receptors, thereby promoting the generation of reactive oxygen species (ROS), which in turn promotes the progression of cardiac hypertrophy [3]. A traditional antioxidant signaling pathway, Nrf2, plays a role in the initiation of cardiac hypertrophy. By regulating antioxidant enzymes, Nrf2 eliminates harmful substances like ROS [4]. Earlier studies demonstrated that AngII treatment increased the levels of p-ERK, p-p38, and p-JNK in mice [5,6]. Blocking the mitogen-activated protein kinases (MAPK) pathway is one of the strategies to improve cardiac hypertrophy [7].

The active ingredients in Chinese medicine are important for inhibiting various pathophysiological processes. Pogostone is the main component of the Chinese herbal medicine *Pogostemon cablin* (Blanco) Benth. It has been reported to have various biological activities such as antioxidant, anti-inflammatory, and immunosuppressive properties. Pogostone has been found to reduce the cellular damage caused by TNF-α, by promoting the activation of Nrf2, inhibiting ROS production, and enhancing the expression of antioxidant genes [8]. Pogostone also ameliorated endotoxic shock in mice by inhibiting the phosphorylation of JNK and p38 MAPK [9]. However, the function of Pogostone in myocardial hypertrophy and related mechanisms are still unclear. This study therefore aimed to investigate the activity of pogostone on Ang II-induced myocardial hypertrophy.

EXPERIMENTAL

Cell culture and treatment

The cells (H9c2) were purchased from the Chinese Academy of Sciences (China, Shanghai) and cultured in DMEM medium with 10 % fetal bovine serum, 1% penicillin : streptomycin (100 IU/mL:100 µg/mL) in a humidified air incubator at 37 °C with 5 % CO_2. Cells were divided into control group, Ang II group, Ang II + pogostone (5 µM) group, Ang II + pogostone (10 µM) group, and Ang II + pogostone (20 µM) group. All groups except the control group were treated with Ang II (1 µM) in H9c2 cells for 24 h [10]. Cells in Ang II + pogostone group were treated with different concentrations of pogostone for 6 h before treatment with AngII.

CCK8 assay

The viability of H9c2 cells was assessed using the CCK8 kit. Cells were cultured in serum-free medium for 4 h, and treated for 24 h with pogostone at concentrations of 5, 10, 20, and 40 µM. The CCK-8 solution (10 µL) was added to each well and incubated for 2 h. Absorbance of cells was determined at 450 nm.

Determination of ROS

Intracellular ROS production was quantified by using a reactive oxygen species assay (ROS) kit. The cells were treated with Ang II or pogostone, washed with PBS, and then incubated with DCFH-DA solution for 20 min in the dark. Following the completion of the assay, fluorescence was measured using fluorescence microscopy.

Determination of MDA

The concentration of MDA in H9c2 cells was assessed following manufacturer's recommendations. Cells were lysed, centrifuged, and the supernatant was collected, mixed with TBA solution and MDA solution, incubated at 95 °C for 1 h, and cooled to room temperature. After the mixture was centrifuged, the absorbance was measured at 532 nm to determine MDA concentration.

Immunofluorescence

Paraformaldehyde (4 %) was used to fix H9c2 cells for 20 min, permeablized for 20 min with 0.5 % Triton X-100 and thereafter blocked with 1 % BSA for 30 min at room temperature. Cells were treated with α-actinin antibody (SCBT, USA, 1 : 100) and incubated at 4 °C. The cells were washed with PBS, incubated with a secondary antibody coupled to FITC, and fluorescent images were observed using fluorescence microscopy. ImageJ software was used to calculate the surface area of the cells, and from at least 50 randomly chosen cells, the surface area of H9c2 cells was calculated [11].

Measurement of apoptosis

The Annexin V-FITC kit was used to measure the apoptosis of H9C2 cells. Cells (1.5×10^5) were seeded in 24-well plates, then washed and resuspended in binding buffer. The cells were treated with 10 µL PI and 5 µL annexin V-FITC for 15 min. Analysis of apoptosis was done by flow cytometry.

Western-blot

Total proteins of H9c2 were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer which was then measured using a BCA

protein assay kit. Protein electrophoresis was performed using 10 % sodium doceyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene diflouride (PVDF) membranes. The membranes were washed with TBST, sealed with 5 % skim milk powder, and incubated with the appropriate primary antibody at 4 °C. The PVDF membranes were washed and incubated with secondary antibody at room temperature. The proteins were developed using the ECL chemiluminescence kit [12-15]. β-actin was used as an internal reference, and protein quantification was performed using ImageJ software. Antibody information was as follows: ANP (Affinity, USA, DF6497, 1:1000), BNP (Affinity, DF6902, 1:1000), Bax (Affinity, AF0120, 1:2000), cleaved-caspase3 (Affinity, AF7022, 1:1000), Nrf2 (Affinity, AF0639, 1:1000), p38 MAPK (Affinity, AF6456, 1:1000), Phospho-p38 MAPK (Affinity, AF4001, 1:1000), Phospho-ERK (Affinity, AF1015, 1:1000), ERK (Affinity, AF0155, 1:1000), Phospho-JNK (Affinity, AF3318, 1:1000), JNK (Affinity, AF6318, 1:1000), β-actin (Affinity, AF7018, 1:5000), IgG (Affinity, S0001, 1:10000).

Statistical analysis

Data analysis was performed using SPSS 22.0, and values expressed as mean ± standard error of the mean (SEM). Determinations were done in triplicate and one-way analysis of variance was used to compare differences between groups. $P < 0.05$ was considered statistically significant.

RESULTS

Pogostone ameliorated Ang II-induced cardiomyocyte hypertrophy

The H9c2 cells were treated with different concentrations of pogostone (Figure 1 A) to prove its effect on cell viability, and results showed that pogostone (5, 10, and 20 μM) did not affect H9c2 cell viability (Figure 1 B). It was discovered that the protein expression of ANP and BNP increased in Ang II-induced H9c2 cells, and α-actinin staining showed an increase in the cardiomyocyte area, whereas treatment with Pogostone decreased the protein expression of ANP and BNP (Figure 1 C) and cardiomyocyte area (Figure 1 E).

Pogostone ameliorated Ang II-induced oxidative stress

The results revealed that Ang II-induced ROS and MDA increase in H9c2 cells, while pogostone (10 and 20 μM) treatment significantly decreased ROS and MDA levels. Cells treated with pogostone (5 μM) had no effect on ROS and MDA levels, indicating that pogostone inhibited Ang II-induced oxidative stress in the cells (Figure 2 A and B).

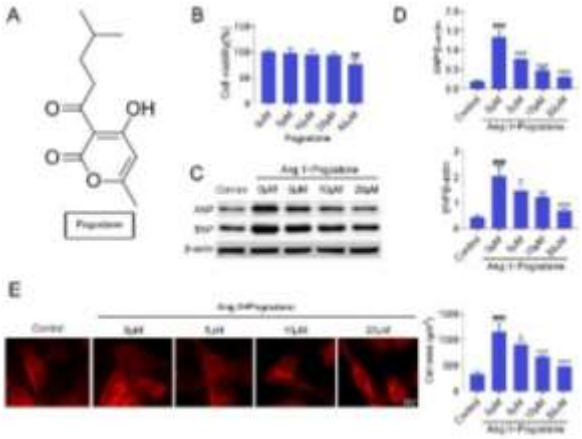

Figure 1: Pogostone ameliorated Ang II-induced cardiomyocyte hypertrophy. (A) Structural formula of pogostone, (B) effect of different concentrations of pogostone on cell viability, (C) pogostone reduces Ang II-induced the protein expression of ANP and BNP, (D) quantification of protein expression of ANP and BNP, (E) pogostone inhibits cardiomyocyte hypertrophy. $^{\#\#}P < 0.01$, $^{\#\#\#}p < 0.001$ vs. control, $^p < 0.05$, $^^p < 0.01$ $^^^p < 0.001$ vs Ang II

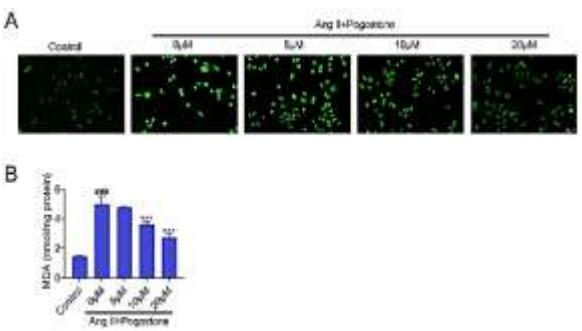

Figure 2: Pogostone ameliorates angiotensin II-induced oxidative stress. (A) Pogostone reduced Ang II-induced ROS production, (B) Pogostone reduced Ang II-induced MDA production. $^{\#\#\#}P < 0.001$ vs control, $^^^p < 0.001$ vs Ang II

Pogostone inhibited Ang II-induced apoptosis

Ang II-induced apoptosis rate and apoptosis-related proteins Bax and cleaved-caspase 3 protein were significantly increased in H9c2 cells. Apoptosis rate (Figure 3 A and B), Bax, and cleaved-caspase3 protein expression (Figure 3 C and D) of cells treated with pogostone (10 and 20 μM) decreased. Pogostone (5 μM) also decreased the protein expressions of Bax, cleaved-caspase3 and apoptosis rate, but there was no significant difference in the effect on the apoptosis rate.

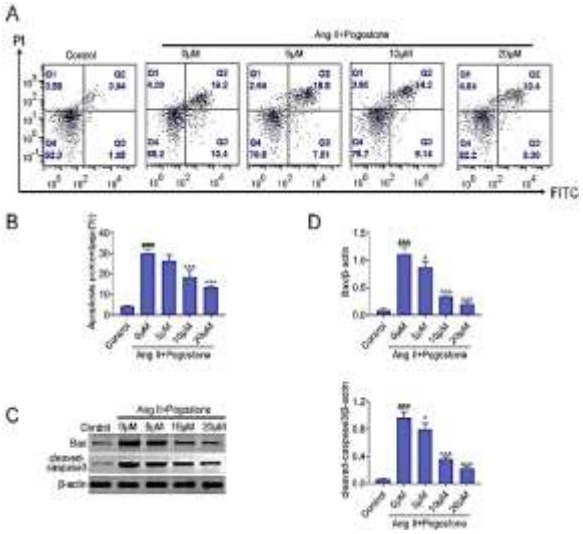

Figure 3: Pogostone inhibited Ang II-induced apoptosis. (A) Pogostone inhibited Ang II-induced apoptosis, (B) quantification of apoptosis rate, (C) pogostone inhibited the expression of Bax and cleaved-caspase3 in cells treated with Ang II, (D) quantification of western blots for Bax and cleaved-caspase3. $^{###}P < 0.001$ vs. control, $^{\wedge}p < 0.05$, $^{\wedge\wedge\wedge}p < 0.001$ vs Ang II

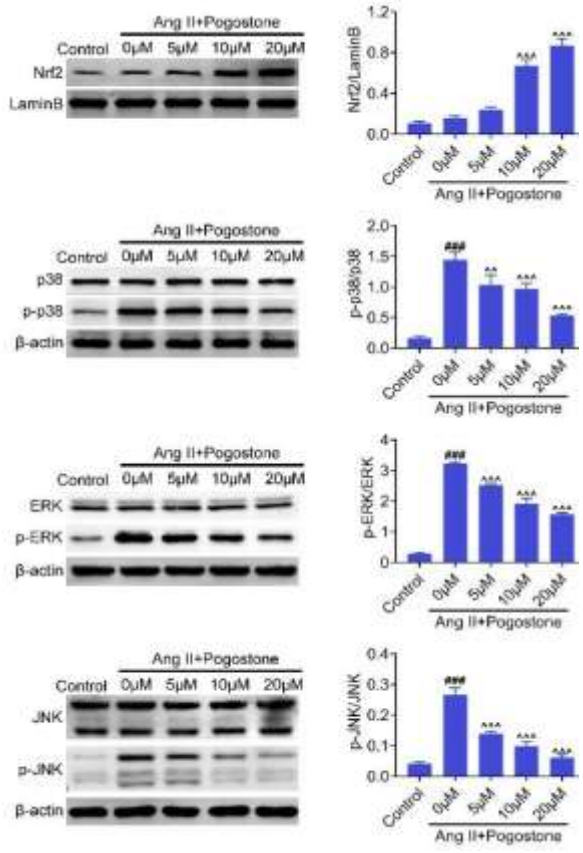

Figure 4: Pogostone regulated Nrf2 and MAPKs pathways. Protein expression of Nrf2, p38, ERK, and JNK. $^{###}P < 0.001$ vs. control; $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs Ang II

Pogostone regulated Nrf2 and MAPKs pathways

Nrf2 protein and the phosphorylation levels of p38, ERK and JNK were increased in H9c2 cells treated with Ang II. Pogostone increased Nrf2 protein expression and decreased the phosphorylation levels of p38, ERK and JNK, indicating that pogostone activated the Nrf2 pathway and inhibited MAPKs pathway (Figure 4).

DISCUSSION

Myocardial hypertrophy, which is characterized by an increase in cardiac cell surface area, results from prolonged overloading of the heart [16]. The natriuretic peptides ANP and BNP, which are secreted by the heart are biologically active peptides frequently employed as prognostic indicators in patients with cardiomyocyte hypertrophy [17]. In this study, H9c2 cells were treated with AngII to establish an *in vitro* model of cardiac hypertrophy. Pogostone treatment ameliorated AngII-induced cardiomyocyte hypertrophy, lowered levels of ANP, BNP, and cell surface area; reduced ROS production and apoptosis. In addition, pogostone activated the Nrf2 pathway and inhibited the MAPKs pathway. This indicates that pogostone has potential therapeutic value for cardiac hypertrophy. When ROS are produced and the innate antioxidant system is unable to sufficiently combat them, oxidative stress occurs. Excessive ROS generation causes lipid peroxidation, and cellular malfunction, and may even cause cellular harm or death. One of the main causes of the growth of cardiac hypertrophy has been linked to ROS. Studies have demonstrated the significant pathogenic role that Ang-II-induced ROS plays in myocardial hypertrophy. As a result, the use of antioxidants as a medicinal strategy is becoming more popular [18]. In this study, pogostone inhibited ROS and MDA production and suppressed apoptosis.

The intracellular redox homeostasis is crucially maintained by Nrf2, a crucial transcription factor in the cellular antioxidant system. Under typical physiological circumstances, Nrf2 is inactively bound to Keap1 in the cytoplasm. When there is oxidative stress, Nrf2 separates from Keap1 and moves to the nucleus [19]. There, it binds to the nuclear ARE and triggers the transcription of genes related to antioxidants [20]. Actually, by stimulating the activity of Nrf2, many herbal substances alleviate myocardial hypertrophy. For instance, Myricetin decreases myocardial hypertrophy by increasing Nrf2 activity [21]. Bitter amygdalin reduces cardiac hypertrophy caused

by AngII by modifying Nrf2 [22]. Previous research has demonstrated that pogostone pretreatment increased Nrf2 expression, protecting against acute lung injury [23]. This study showed that pogostone activated Nrf2 expression thus, acting as an antioxidant. Reactive oxygen species regulate a number of molecular signaling pathways linked to cardiac hypertrophy. Recent research indicated that MAPK signaling pathway, a traditional mechanism involved in oxidative stress-induced hypertrophy, is largely activated by ROS generation [24]. In earlier studies, pogostone alleviated metabolic diseases associated with obesity by inhibiting MAPKs pathway [25]. This study also demonstrated that pogostone reduced the phosphorylation of p38, ERK, and JNK in cells caused by Ang-II. This indicated that pogostone inhibited MAPKs pathway by reducing ROS which is in tandem with previous studies [25].

Limitations of this study

No *in vivo* experiments were performed. Future studies should include *in vivo* research data to validate these findings.

CONCLUSION

Pogostone inhibits oxidative stress, apoptosis, and ameliorates Ang-II-induced cardiac hypertrophy. Furthermore, pogostone promotes the activation of Nrf2 pathway and the inhibition of MAPKs pathway. This study provides a potential strategy for the treatment of myocardial hypertrophy.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ying Yang and Xuebo Liu designed the study and carried them out; Ying Yang, Yuan Xie, Xinna Zhao, and Meiyan Qi supervised the data collection, analyzed the data, and interpreted the data; Ying Yang and Xuebo Liu prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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