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ORIGINAL RESEARCH

Wedelolactone attenuates angiotensin II-stimulated hypertrophy in H9C2 cardiomyocytes

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Abstract

Cardiomyocyte hypertrophy is the adaptive response of the heart to various physiological or pathological stresses. To combat this type of disease, more effective drugs are still needed. Wedelolactone is the main ingredient extracted from the medicinal plant Daylily and have effects such as immunomodulatory, anti-fibrosis, and anti-inflammation. However, the role and mechanism of Wedelolactone in myocardial hypertrophy are still unclear. Here, H9C2 cells were treated with Ang II to construct a cardiomyocyte hypertrophy cell model. We found Wedelolactone suppressed the survival of Ang II-induced cardiomyocytes. Wedelolactone further restrained the progression of Ang II-induced cardiomyocyte hypertrophy. Wedelolactone suppressed the oxidative stress of Ang II-induced cardiomyocytes, and restrained the apoptosis. Mechanically, Wedelolactone restrained the progression of Ang II-induced cardiomyocyte hypertrophy *via* Nuclear factor erythroid 2-related factor 2/Heme oxygenase-1 pathway (Nrf2/HO-1 pathway). In summary, Wedelolactone attenuates angiotensin II-induced hypertrophy and apoptosis in cardiomyocytes. Wedelolactone attenuates angiotensin II-stimulated hypertrophy and apoptosis in cardiomyocytes.

Keywords

Cardiomyocyte hypertrophy; Wedelolactone; Oxidative stress; Apoptosis; Nrf2/HO-1

1. Introduction

Cardiomyocyte hypertrophy includes hypertrophic cardiomyopathy, hypertensive heart disease, aortic stenosis, pulmonary hypertension, *etc.* [1]. This disease can cause heart dysfunction, endangering the health and life of patients, and it is also a common case of severe illness [2]. Due to the aging of the population and changes in risk factors, the incidence of cardiovascular diseases (CVDs) has increased [3]. Pathological myocardial hypertrophy is vital and an independent predictor of CVDs [4]. The pathogenesis of myocardial hypertrophy is complex, which may be related to hypertension, inflammation, oxidative stress and other factors.

Cardiomyocyte hypertrophy is an adaptive response phenotype of the heart to various stresses, leading to the remodeling of the heart to maintain function [4]. Persistent cardiac obesity can alter systolic function, eventually leading to heart failure and sudden death [5]. However, increased oxidative stress has long been considered a major factor in accelerating ventricular hypertrophy [6]. Ang II plays an important role in the regulation of vascular function and it can promote the pathogenesis of CVDs through its stimulated oxidative stress [7]. By stimulating its type 1 receptor, Ang II affected cardiac dysfunction and cell hypertrophy [8]. To combat this type of disease, more effective drugs are still needed.

Wedelolactone is the main ingredient extracted from a medicinal plant which had immunomodulatory, and anti-fibrosis effects [9–11]. Wedelolactone alleviates podocyte inflammation and oxidative stress damage [12]. Wedelolactone restrained Interleukin-1 beta (IL-1β) maturation and neutrophil infiltration [13]. Wedelolactone alleviates Parkinson's disease by blocking oxidative stress and mitochondrial disorder *via* Nuclear factor erythroid 2-related factor 2 (Nrf2)/SKN-1 axis [14]. It also improved synovial inflammation and cardiac complications in a mouse model of collagen-stimulated arthritis [10]. However, the role and mechanism of Wedelolactone in myocardial hypertrophy remain unclear.

Here, H9C2 cells were incubated with Ang II to construct a cardiomyocyte hypertrophy cell model. We revealed that Wedelolactone reduced Ang II-stimulated hypertrophy and oxidative stress in cardiomyocytes *via* the Nrf2/HO-1 axis. We thought Wedelolactone could serve as a potential drug for cardiomyocyte hypertrophy treatment.

2. Materials and methods

2.1 Cell culture and treatment

H9C2 cells were purchased from American Type Culture Collection (ATCC, USA). H9C2 cells were cultured with the Dulbecco's Modified Eagle Medium (DMEM, Gbico, USA) with 10% Fetal Bovine Serum (FBS, Gbico, CA, USA) in a

5% CO_2 incubator at 37 °C and incubated with Ang II (1 μM, HY-P72828, Bought from MCE, NJ, USA) or Wedelolactone (W124219, Bought from sigma, St. Louis, MO, USA) for 24 h at the concentration of 0, 1, 2, 4 μM and maintained at 37 °C for 24 h.

2.2 Cell viability assays

H9C2 cells were seeded into 96-well plates (1000/cell) at 37 °C upon the indicated treatment for 24 h. Cells were treated with cell counting kit-8 (CCK-8) reagent (C0078, Beyotime, Beijing, China) at 37 °C for 3 h. The relative cell viability was assessed by Optical Density (OD) 450 value. After calculating the absorbance value, the value of the control group is normalized to 1, and then the relative value of the experimental group is calculated to reflect the degree of proliferation.

2.3 Phalloidin staining assay

The H9C2 cells were fixed with 4% paraformaldehyde (PFA), blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline with Tween 20 (PBST), and then incubated with Rhodamine-labelled phalloidin (1:2000, ab176757, Abcam) for 20 minutes. After washing with PBST, cell nuclei were labelled with 4′,6-diamidino-2-phenylindole (DAPI) and photographed.

2.4 Reactive Oxygen Species (ROS) assay

The cellular ROS level was determined using Dichlorofluorescein (Sigma-Aldrich). Then cells were washed. Then cells were analyzed under a microplate.

2.5 Flow cytometer (FCM) assay

The H9C2 cells were washed with Phosphate-Buffered Saline (PBS) and fixed using 70% ethanol at −20 °C for 2 h. Subsequently cells were stained with Propidium Iodide (PI) at 4 °C. Then cells were measured using Flow cytometer (BD, Franklin Lakes, NJ, USA).

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

Malondialdehyde (MDA) was detected *via* ELISA (S0131S, Beyotime, Beijing, China). Standard reagents and samples were added and incubated for 20 h at 4 °C. The OD450 value was measured through a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA).

2.7 Immunoblot

The cells upon the indicated treatment for 24 h were electrophoresed by 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), transferred onto Polyvinylidene Fluoride (PVDF) membranes, then blocked with 5% fat-free milk. Subsequently, membranes were incubated with anti-Bax (1:1000, ab32503, Abcam, Cambridge, UK), cleaved caspase-3 (1:1000, ab32042, Abcam), Nrf2 (1:1000, ab62352, Abcam), HO-1 (1:500, ab189490, Abcam), and β-actin (1:3000, ab8226, Abcam) antibodies for 1 h. Ultimately, the membranes were conjugated with the HRP-labelled IgG (Abcam) for 1 h and visualized with Enhanced Chemiluminescence kit (Thermo, Rockford, IL, USA).

2.8 Statistics

GraphPad 5.0 software (GraphPad Software, San Diego, CA, USA) was used and performed for statistical analysis. Data were represented as mean $\pm$ standard deviation (SD), and $p < 0.05$ was thought as significant.

3. Results

3.1 Wedelolactone suppressed the viability of Ang II-stimulated cardiomyocytes

We first constructed a cell model of cardiomyocyte hypertrophy using Ang II-treated H9C2 cells. To detect the effects of Wedelolactone on the viability of Ang II-treated cardiomyocytes, CCK-8 assays were conducted. We first found that the treatment of Wedelolactone (1, 2 and 4 μM) had modest effects on the viability of H9C2 cells (Fig. 1a). However, we noticed that Ang II treatment suppressed the viability of H9C2 cells, and Wedelolactone treatment significantly contributed to the viability of Ang II-stimulated H9C2 cells, with the increased OD450 value (Fig. 1b). Therefore, Wedelolactone suppressed the viability of Ang II-stimulated cardiomyocytes.

3.2 Wedelolactone further restrained the progression of Ang II-stimulated cardiomyocyte hypertrophy

Subsequently, we detected the effects of Wedelolactone on the cardiomyocyte hypertrophy of Ang II-stimulated cardiomyocytes *via* phalloidin staining. Phalloidin staining is known to show the cell contours and indicate hypertrophic cardiomyocytes. We noticed that Ang II treatment obviously increased the degree of cardiomyocyte hypertrophy in H9C2 cells (Fig. 2). However, Wedelolactone treatment suppressed the percentage of hypertrophic cardiomyocytes in Ang II-stimulated H9C2 cells (Fig. 2). Therefore, Wedelolactone restrained the progression of Ang II-stimulated cardiomyocyte hypertrophy.

3.3 Wedelolactone suppressed the oxidative stress of Ang II-stimulated cardiomyocytes

We further explored whether Wedelolactone affected the oxidative stress of Ang II-stimulated cardiomyocytes. Through the DCF staining, we found that Ang II treatment obviously increased the DCF intensity in H9C2 cells, suggesting the promoting of oxidative stress (Fig. 3a). However, Wedelolactone suppressed the DCF staining in Ang II-stimulated H9C2 cells (Fig. 3a). We further detected the levels of MDA, which reflects the degree of oxidative stress, in Ang II-stimulated H9C2 cells. We noticed that Ang II treatment increased the levels of MDA in H9C2 cells, whereas Wedelolactone further suppressed the levels of MDA in Ang II-stimulated H9C2 cells (Fig. 3b). Therefore, Wedelolactone suppressed the oxidative stress of Ang II-stimulated cardiomyocytes.

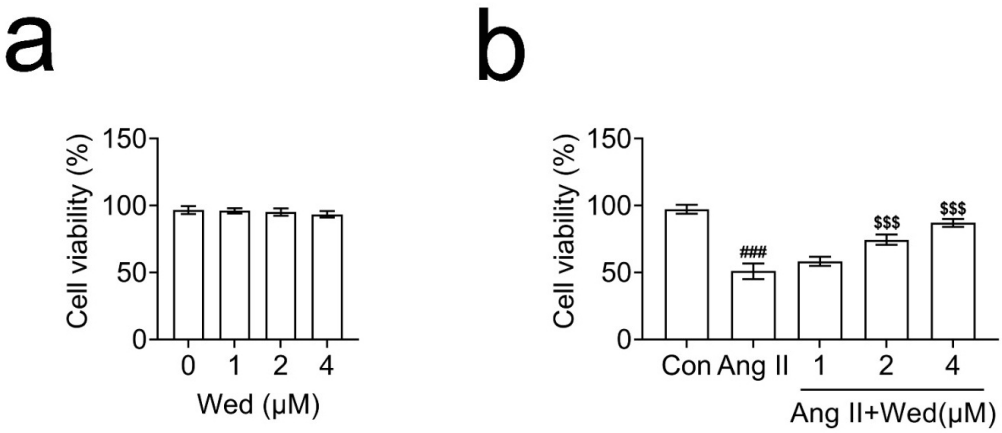

FIGURE 1. Wedelolactone suppressed the viability of Ang II-stimulated cardiomyocytes. (a) CCK-8 assays showed the effects of Wedelolactone (Wed) on the viability of H9C2 cells at the concentration of 1, 2 and 4 μM for 24 h. The OD450 value was measured. (b) CCK-8 assays showed the effects of Wedelolactone (Wed) on the viability of Ang II-stimulated H9C2 cells at the concentration of 1, 2 and 4 μM for 24 h. The OD450 value was measured. Wed, Wedelolactone; Ang II, Angiotensin II; Con, Control. ###$p < 0.001$, Ang II *vs.* control, \$\$\$$p < 0.001$, Ang II + Wed *vs.* Ang II.

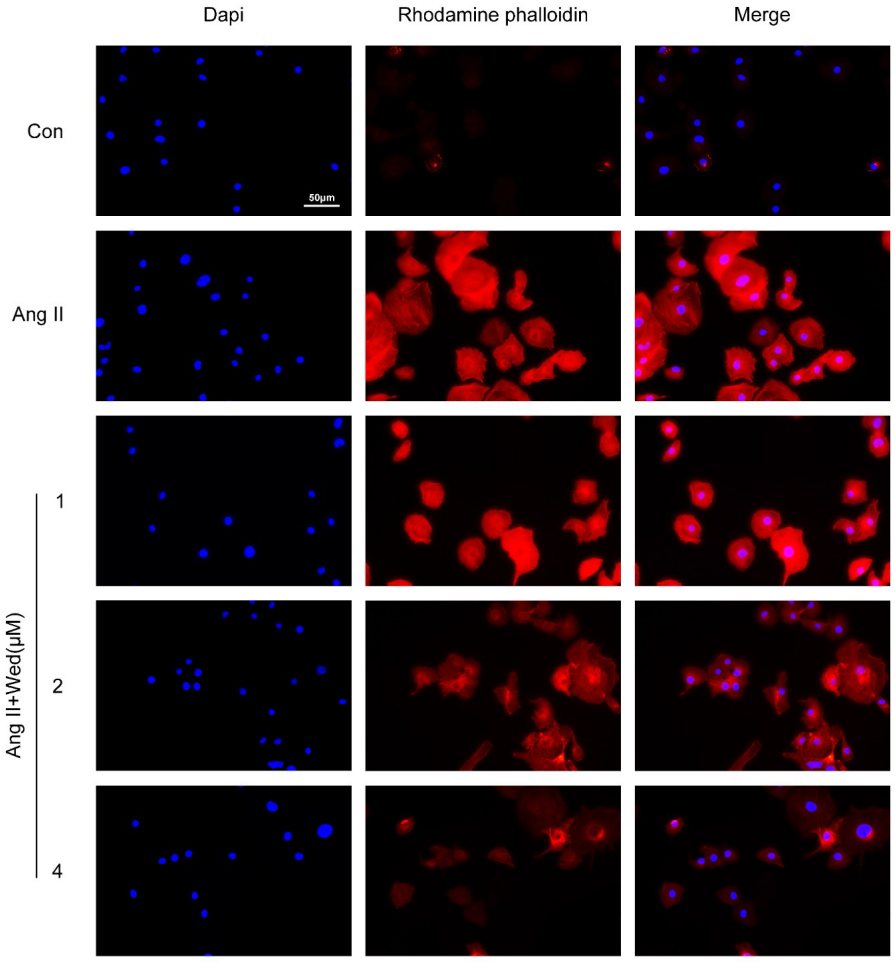

FIGURE 2. Wedelolactone further restrained the progression of Ang II-stimulated cardiomyocyte hypertrophy. Phalloidin staining assays showed the staining degree of phalloidin in H9C2 cells upon Ang II treatment and Wedelolactone (Wed) treatment at the concentration of 1, 2 and 4 μM for 24 h. Red panel indicates phalloidin. Ang II, Angiotensin II; Con, Control.

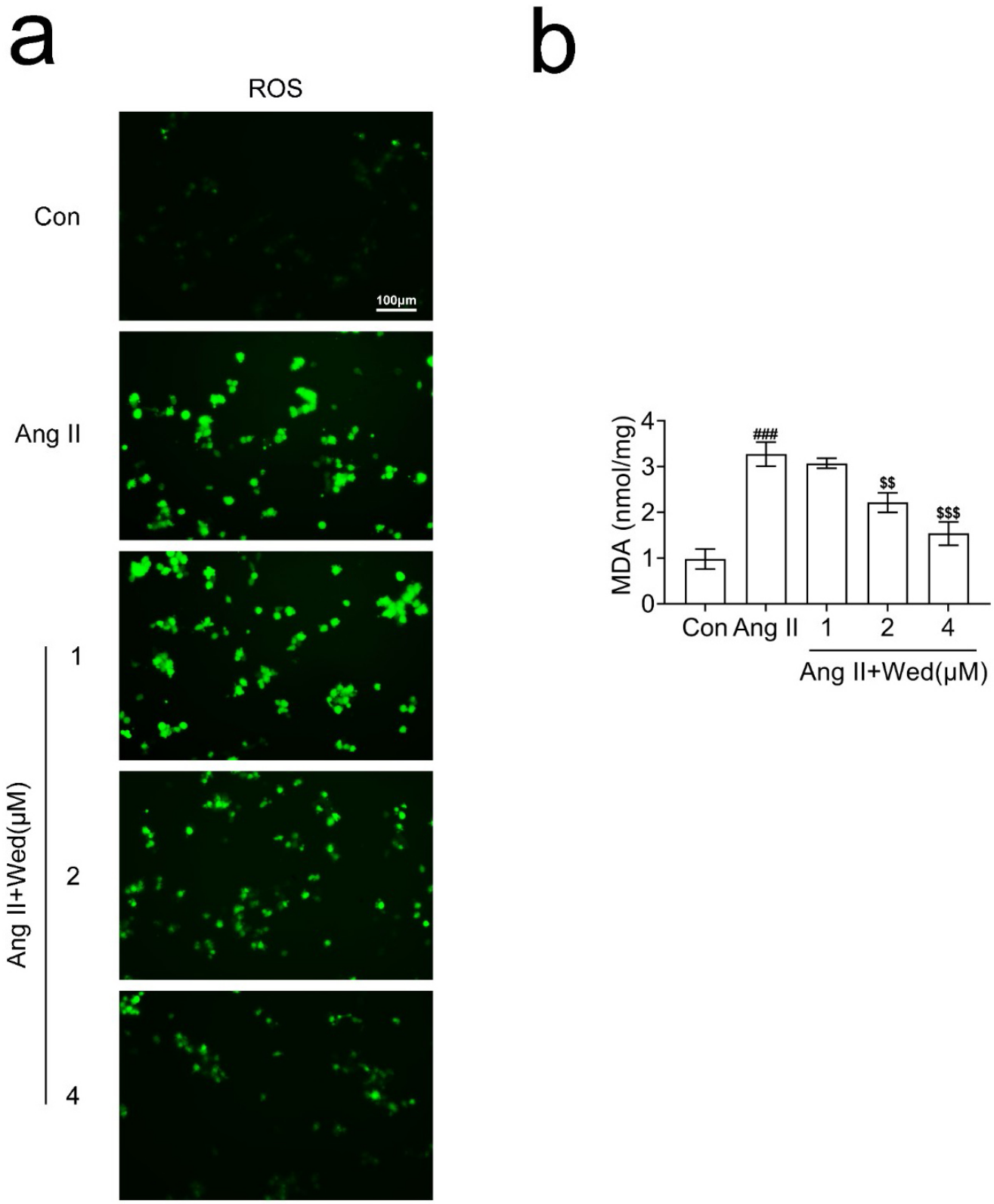

FIGURE 3. Wedelolactone suppressed the oxidative stress of Ang II-stimulated cardiomyocytes. (a) DCF staining assays showed the degree of oxidative stress in H9C2 cells upon Ang II treatment and Wedelolactone (Wed) treatment at the concentration of 1, 2 and 4 μM for 24 h. Green panel indicates DCF levels. Scale bar, 100 μM. (b) ELISA showed the MDA levels in H9C2 cells upon Ang II treatment and Wedelolactone (Wed) treatment at the concentration of 1, 2 and 4 μM for 24 h. Wed, Wedelolactone; Ang II, Angiotensin II; Con, Control; ROS, Reactive Oxygen Species; MDA, Malondialdehyde, ###$p < 0.001$, Ang II *vs.* control, \$\$$p < 0.01$, \$\$\$$p < 0.001$, Ang II + Wed *vs.* Ang II.

3.4 Wedelolactone blocked the apoptosis of Ang II-stimulated cardiomyocytes

We then detected the effects of Wedelolactone on the apoptosis of Ang II-stimulated cardiomyocytes. Through FCM assays, we revealed that Ang II stimulated the apoptosis of H9C2 cells (Fig. 4a). Whereas Wedelolactone treatment suppressed the apoptosis of Ang II-stimulated H9C2 cells (Fig. 4a). Consistently, immunoblot assays confirmed that Ang II increased the expression of Bax and cleaved caspase-3, the markers of apoptosis, in H9C2 cells, suggesting the promoting of apoptosis (Fig. 4b). Wedelolactone treatment decreased the expression of these markers (Fig. 4b). Collectively, Wedelolactone inhibited the apoptosis of Ang II-stimulated cardiomyocytes.

3.5 Wedelolactone mediated Nrf2/HO-1 pathway in Ang II-stimulated cardiomyocytes

Finally, the underlying mechanism was explored. Immunoblot assays showed that Ang II treatment increased the expression of Nrf2 and HO-1 in H9C2 cells, whereas Wedelolactone treatment further increased the expression of Nrf2 and HO-1 in H9C2 cells upon the treatment of Ang II, suggesting that Wedelolactone mediated Nrf2/HO-1 pathway in Ang II-stimulated cardiomyocytes (Fig. 5). Therefore, Wedelolactone mediated Nrf2/HO-1 pathway in Ang II-stimulated cardiomyocytes.

4. Discussion

Pathological cardiac hypertrophy is an injury reaction that occurs when the heart is overloaded [7]. It is mainly manifested by the increase of cardiomyocyte volume, interstitial and perivascular fibrosis, loss of cardiomyocytes, increase in collagen synthesis and activation of myofibroblasts, which eventually lead to the disturbance of myocardial structure, the decreased contractility, and the dysfunction of myocardial contraction and diastole [2]. Pathological myocardial hypertrophy is an independent risk factor of CVDs [2]. Physical stimulation such as exercise or hyperthyroidism, hypertension and other pathologic stimulation can induce physiological or pathological myocardial hypertrophy [4]. Cellular redox imbalance or mitochondrial dysfunction is one of the important mechanisms of myocardial hypertrophy [15]. Here, we constructed a cell model of cardiac hypertrophy, and revealed that Wedelolactone activated the Nrf2 pathway and improved Ang II-stimulated cardiomyocyte hypertrophy. We therefore thought Wedelolactone could serve as a promising drug for cardiomyocyte hypertrophy.

Wedelolactone, a primary active component which exhibits a range of biological activities [12]. This compound is recognized for its immunomodulatory properties and it also has anti-fibrotic and anti-inflammatory effects [10, 12, 13]. Additionally, it has capabilities to counteract free radicals, contributing to its antioxidative properties, and plays a role in enhancing physical stamina and alleviating fatigue [16]. Its primary effect is to mitigate the vasodilation and increased vascular permeability caused by histamine, thereby alleviating symptoms such as redness and itching associated with allergic reactions [17, 18]. In therapeutic use, these cardiac effects are generally not a major clinical concern. Interestingly, we here revealed that Wedelolactone suppressed the oxidative stress and apoptosis of Ang II-stimulated cardiomyocytes.

Oxidative stress, caused by free radicals, can cause cellular damage and trigger pathological changes in cardiac cells, thereby exacerbating myocardial hypertrophy [19]. This stress activates various cellular signaling pathways [20]. On the other hand, apoptosis, or programmed cell death, increases during myocardial hypertrophy, negatively impacting heart function and structure [21, 22]. Thus, these two mechanisms collectively drive the progression of myocardial hypertrophy and may become potential targets for treatment. Here, we revealed that Wedelolactone reduced Ang II-stimulated oxidative stress and apoptosis, therefore suppressing the progression of cardiomyocyte hypertrophy.

The Nrf2/HO-1 pathway is crucial in defending against myocardial hypertrophy [23]. Nrf2 is a transcription factor that controls the expression of antioxidant proteins, and HO-1 is one of these enzymes [24]. Activation of this pathway can reduce oxidative stress, a key player in myocardial hypertrophy [23]. By alleviating oxidative damage and inflammation, the Nrf-2/HO-1 pathway offers therapeutic promise for controlling or preventing cardiac hypertrophy [24]. Several drugs suppressed the progression of cardiomyocyte hypertrophy *via* this pathway. For example, Wogonin, a natural agent, has been shown to have potential therapeutic value in myocardial hypertrophy by activating the Nrf-2-mediated antioxidant responses [25]. Wogonin treatment significantly inhibited oxidative stress. Interestingly, we here revealed that Wedelolactone reduced Ang II-stimulated cardiomyocyte hypertrophy in cardiomyocytes *via* the Nrf2/HO-1 pathway, further confirming that this pathway is vital in combating cardiomyocyte hypertrophy.

A previous study indicated that Wedelolactone protects human bronchial epithelial cell injury against cigarette smoke extract-induced oxidant stress and inflammation responses through Nrf2 pathway [24]. Moreover, another study found that Wedelolactone mitigates parkinsonism *via* alleviating oxidative stress and mitochondrial dysfunction through Nrf2/SKN-1 axis [23]. These studies all revealed that the regulation of Nrf2 by Wedelolactone occurred in different diseases. Given the fact that Nrf2 mainly regulates iron death and has regulatory effects on different cellular processes, we believe that Wedelolactone suppressed angiotensin II-stimulated hypertrophy in H9C2 cardiomyocytes through Nrf2. Our study reveals the role of Wedelolactone in a new disease model. However, the underlying molecular mechanisms still require further investigation.

The limitation of this study is that the relevant experiments were carried out at the cellular level, and only the effect of Wedelolactone on myocardial mast cell models was confirmed at the cellular level. Next, we will conduct animal experiments to verify the effects of Wedelolactone on the phenotype of myocardial hypertrophy in mice models of myocardial hypertrophy. Another limitation of this study is the lack of detailed mechanism studies. We intend to carry out multi-omics to reveal downstream proteins regulated by Wedelolactone in myocardial mast cell models, thereby revealing more about its mechanism.

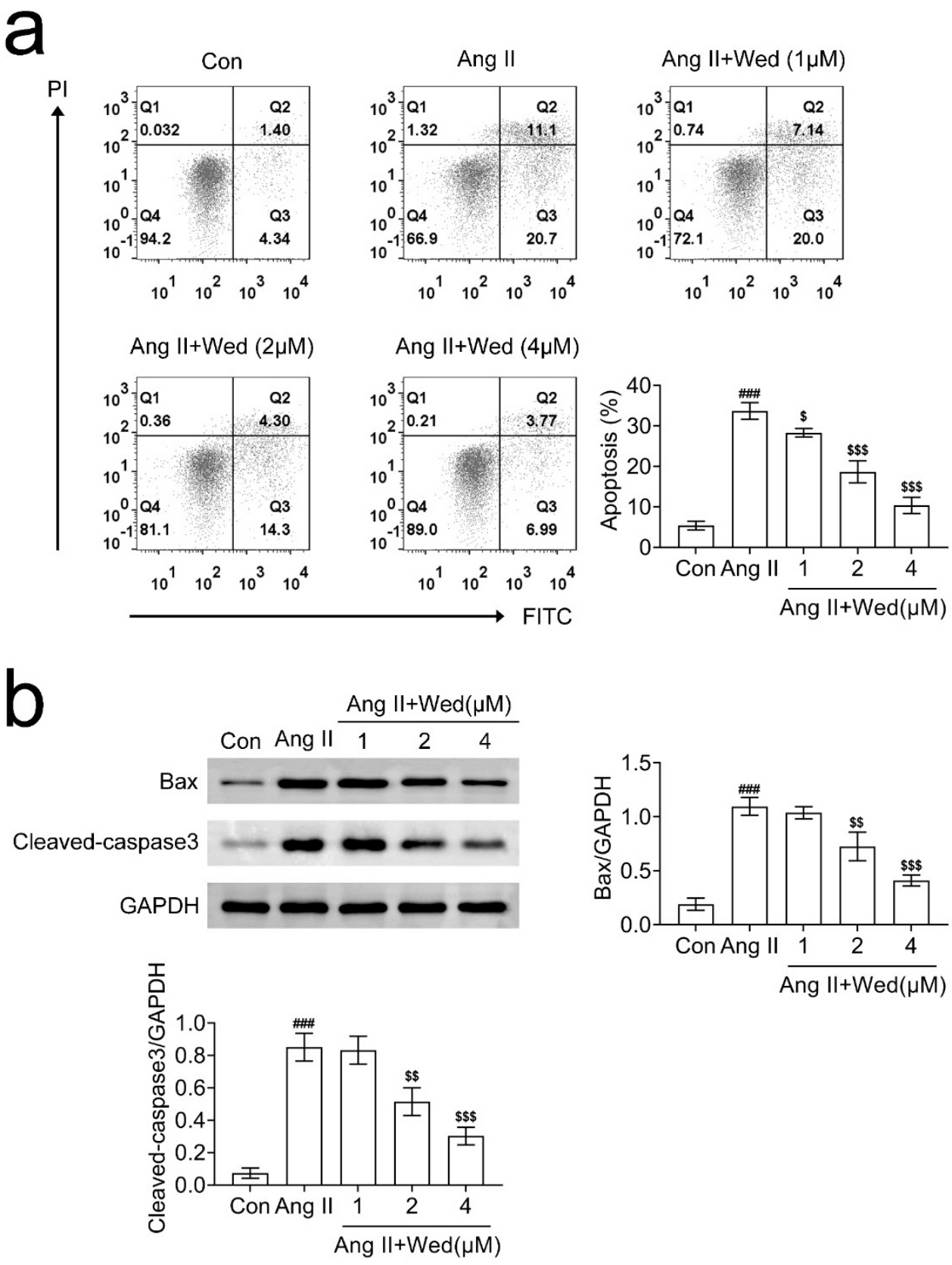

FIGURE 4. Wedelolactone blocked the apoptosis of Ang II-stimulated cardiomyocytes. (a) FCM assays showed the apoptosis cell percentage of H9C2 cells upon Ang II treatment and Wedelolactone (Wed) treatment at the concentration of 1, 2 and 4 μM for 24 h. (b) Immunoblot showed the expression of Bax and cleaved caspase-3 in H9C2 cells upon Ang II treatment and Wedelolactone (Wed) treatment at the concentration of 1, 2 and 4 μM for 24 h. Wed, Wedelolactone; Ang II, Angiotensin II; Con, Control; PI, Propidium Iodide; FITC, Fluorescein Isothiocyanate; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase. ###$p < 0.001$, Ang II *vs.* control, \$$p < 0.05$, \$\$$p < 0.01$, \$\$\$$p < 0.001$, Ang II + Wed *vs.* Ang II.

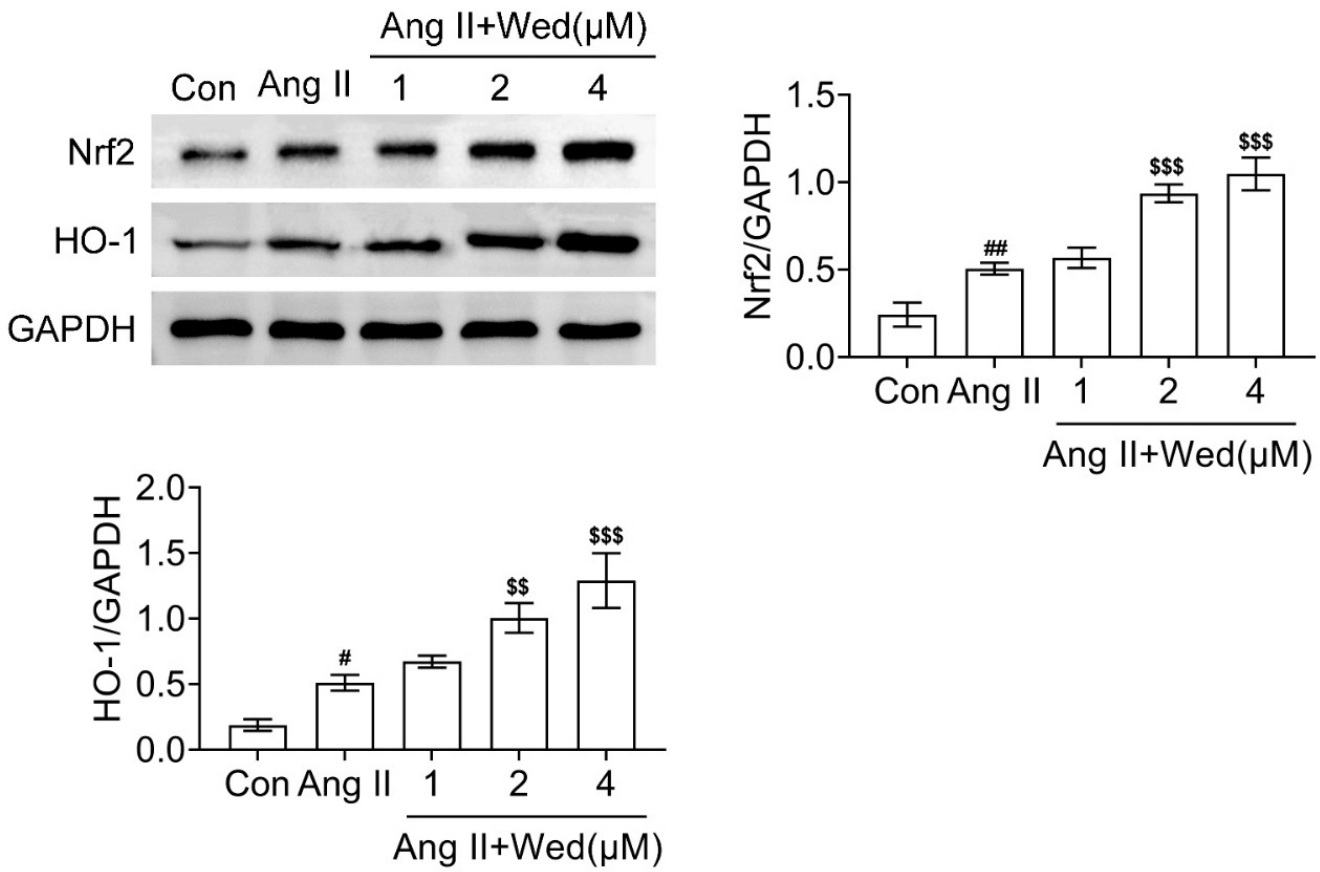

FIGURE 5. Wedelolactone mediated Nrf2/HO-1 pathway in Ang II-stimulated cardiomyocytes. Immunoblot showed the expression of Nrf2 and HO-1 in H9C2 cells upon Ang II treatment and Wedelolactone (Wed) treatment at the concentration of 1, 2 and 4 μM for 24 h. The relative levels of Nrf2 and HO-1 were quantified. Wed, Wedelolactone; Ang II, Angiotensin II; Nrf2, Nuclear factor erythroid 2-related factor 2; HO-1, Heme oxygenase-1. #$p < 0.05$, ##$p < 0.01$, Ang II *vs.* control, \$\$$p < 0.01$, \$\$\$$p < 0.001$, Ang II + Wed *vs.* Ang II.

5. Conclusions

In conclusion, Wedelolactone attenuates angiotensin II-stimulated hypertrophy, oxidative stress, and apoptosis in H9C2 cardiomyocytes. Therefore, Wedelolactone could serve as a promising drug for myocardial hypertrophy.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

AUTHOR CONTRIBUTIONS

RFZ and LLJ—designed the study, completed the experiment and supervised the data collection. DX—analyzed the data, interpreted the data. CB and JJH—prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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