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Original Article

The attenuation of myocardial hypertrophy by atorvastatin via the intracellular calcium signal and the p38 MAPK pathway

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Abstract: Objective: It is well documented that atorvastatin could protect against atherosclerosis, cardiac fibrosis, etc. However, few reports have drawn the link between atorvastatin and myocardial hypertrophy, a common type of myocardial damage. This study aimed to illustrate the effects of atorvastatin on Ang II-induced cardiac hypertrophy and to reveal its mechanism. Methods: We established cardiac hypertrophy by exposing cardiomyocytes to Ang II. Then we determined whether atorvastatin could reverse cardiac hypertrophy markers and several cellular responses induced by Ang II to normal levels. Finally, we tried to illustrate the mechanism of these effects. Results: Atorvastatin performed very well in resuming cardiac hypertrophy. It could attenuate the increase of oxidative stress and cell apoptosis in cardiomyocyte cells. The activation of the p38 MAPK signaling pathway induced by Ang II was well inhibited by atorvastatin. Additionally, the Ca^{2+} concentration in cells and the calcineurin (CaN) expression level were also significantly mitigated by atorvastatin. Conclusion: Atorvastatin can attenuate cardiac hypertrophy induced by Ang II via the intracellular calcium signal and the p38 MAPK pathway. It provides a therapeutic potential for the treatment of myocardial hypertrophy.

Keywords: Atorvastatin, cardiomyocytes, p-38 MAPK signaling pathway, Ca^{2+}

Introduction

Cardiac hypertrophy, which is associated with myocardial fibrosis, capillary rarefaction, inflammatory reaction, and cellular dysfunction, which can consequently cause maladaptive ventricular remodeling and heart failure. The etiology of myocardial hypertrophy may be ascribed to external stimulation, such as mechanical stress or neurohumors. Several cellular responses, including gene transcription, protein translation and cell metabolism have been shown to be associated with the progression of myocardial hypertrophy [1, 2]. Multiple studies have proved that angiotensin II (Ang II) is a critical factor in the development of cardiac hypertrophy, and thus, heart failure may result [3, 4]. Numerous studies have also revealed that Ang II could induce cardiac hypertrophy by the AT 1 receptor, inducing oxidase activation and the over-production of ROS [5, 6]. The increasing oxidative stress would induce mitochondrial dysfunction, cell apoptosis, autophagy, intracellular disorder and then lead to cardiac dysfunction [7-11].

In recent years, many signal pathways have been also proved to be related to cardiac hypertrophy [12, 13]. Among these, calcineurin, calcium/calmodulin-activated serine-threonine phosphatase, is the key factor in cardiac hypertrophy [14]. The overexpression of calcineurin can cause pathological cardiac hypertrophy in mice. In contrast, the down-regulation of calcineurin could protect the heart from cardiac hypertrophy [15]. Additionally, a growing number of studies have revealed that the over-activation of the p-38 MAPK signaling pathway could trigger heart disease. The inhibition of p-38 MAPK can attenuate cardiac fibrosis in mice [16, 17].

Atorvastatin can alleviate cholesterol in the blood by inhibiting HMG-CoA reductase, thus leading to blood cholesterol dysregulation and

Table 1. Primers used for the quantitative PCR analysis

Gene name	Primer sequence
Gapdh	Forward 5' AAGAAGGTGGTGAAGCAGGC-3' Reverse 5' TCCACCACCCTGTTGCTGTA-3'
ANP	Forward 5'-CTCCGATAGATCTGCCCTCTTGAA-3' Reverse 5'-GGTACCGGAAGCTGTTGCAGCCTA-3'
β-MHC	Forward 5'-CCAGAAGCCTCGAAATGTC-3' Reverse 5'-CTTTCTTTGCCTTGCCTTTGC-3'
BNP	Forward 5'-TGATTCTGCTCCTGCTTTTC-3' Reverse 5'-GTGGATTGTTCTGGAGACTG-3'
	Reverse 5' TCTGCGGATCTTGGACAAACAA-3'

a reduced risk of cardiovascular disease. The mechanism of atorvastatin in reducing blood cholesterol is its function of regulating the lipoprotein-cholesterol complex and liver function [18]. Also, atorvastatin has been applied to treat many heart diseases, such as cardiovascular disease and atherosclerosis, due to its modulating the function of some cells, such as endothelial cells, or some pathways such as the ROCK pathway [19, 20]. However, there are few studies about the role of atorvastatin in cardiac hypertrophy, even though it has been used in many heart disease treatments. Additionally, the mechanism remains unknown.

In this contribution, we aimed to illustrate the effect of atorvastatin on Ang II-induced cardiac hypertrophy. We established the cardiac hypertrophy cell model utilizing cardiomyocyte cells. Then we determined several cellular responses induced by Ang II, such as oxidative stress, cell apoptosis, the activation of the p-38 MAPK signaling pathway, and the calcineurin level, which were all corelated with cardiac hypertrophy. Our data suggest that atorvastatin could be an efficient ancillary drug for the therapy of cardiac hypertrophy.

Materials and methods

Materials

Ang II was purchased from Sigma (St. Louis, MO, USA). Fluo-3/AM (5 μM) was obtained from Molecular Probes (Molecular Probes, USA). Atorvastatin was purchased from Pfizer (New York, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a ROS detection kit, and a TUNEL staining kit were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). Anti-ANP, anti-BNP, anti-p38, anti-JNK, anti-BCL-2, anti-Caspase-3, and anti-calcineurin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-BAX (Clone 6A7) was obtained from Thermo Scientific (MA, USA). Anti-phospho-p38, anti-phospho-JNK, and anti-β-MHC were purchased from Cell Signaling Technology (Cell Signaling Tech, Boston, USA). Malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) detection kits were purchased from Jiancheng Biotechnology (Jiancheng Biotech, China). TRIzol reagent was acquired from Takara (Takara Biochemicals, Dalian, China). The ReverTra Ace® qPCR RT Kit and the SYBR® Green Realtime PCR Master Mix were obtained from Toyobo (Tokyo, Japan). All oligonucleotide primers were synthesized in Sangon Biotech (Sangon, Shanghai, China).

Cell culture

Wistar rats were purchased from the Experimental Animal Center of Harbin Medical University. The rats then were cared for in accordance with the *Guiding Principles in the Use of Animals* at the Second Hospital of Jilin University. Myocardial cells were isolated from 1-day-old neonatal Wistar rat hearts [21]. The cells were cultured under standard cell culture conditions in a DMEM medium culture (Invitrogen, Carlsbad, CA), and then supplemented with 10% heat-inactivated FBS (Gibco, USA) and 1% penicillin-streptomycin (Thermo Scientific). Before starting the experiments, the cells were pre-cultured until confluence was reached. Ang II was added into the culture medium at a final concentration of 0.1 μM for 48 h to induce cell hypertrophy. Atorvastatin (50 μM) exposure was performed 3 h before the Ang II treatment. The cell image was taken by microscopic examination (SDPTOP ICX41, China). The cell surface area was calculated from the number of pixels by Image-Pro Plus (version 5.0.1).

Quantitative real-time PCR analysis

For the quantitative analysis of the genes, the cardiomyocytes were seeded in 12-well plates at a density of 10×10^4 cells per well. The cells were treated as previously described for 48 h. The mRNA levels of *ANP*, *BNP* and *β-MHC* were quantified by semi-quantitative real-time PCR analysis (RT-qPCR). Total RNA was isolated from the myocardial cells using the TRIzol

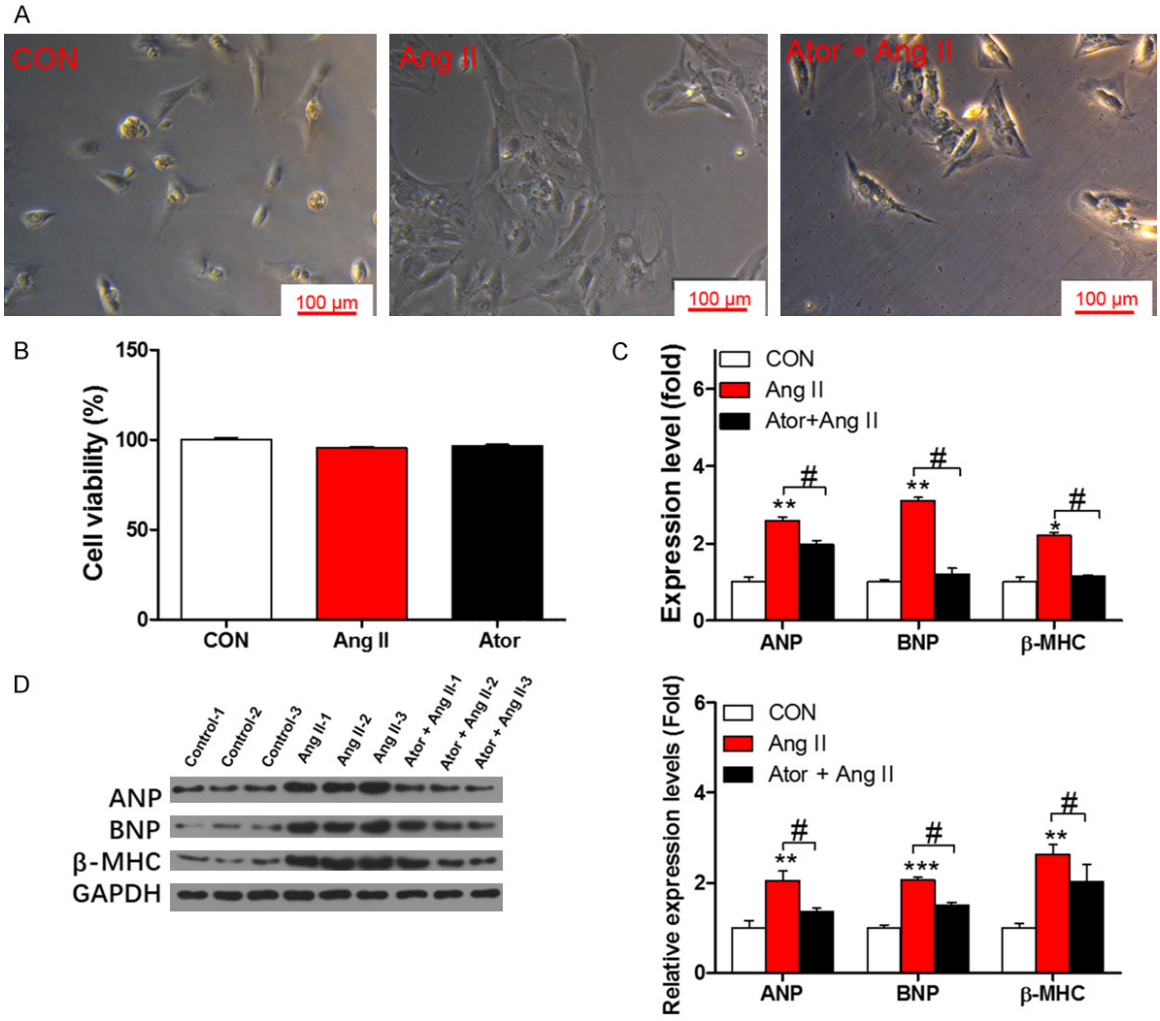

Figure 1. Atorvastatin attenuated Ang II-induced cardiac hypertrophy. A. Effects of Ang II and Atorvastatin on cardiomyocytes morphology. B. Cardiomyocyte cell viability after 48 h treatment with Ang II (0.1 μM) or atorvastatin (50 μM). C. Effects of Ang II and Atorvastatin on the mRNA levels of ANP, BNP and β-MHC. D. Effects of Ang II and Atorvastatin on the protein levels of ANP, BNP and β-MHC. Data were expressed as the mean ± SEM. n = 3. **P < 0.05*, ***P < 0.01* compared with the control group, *#P < 0.05* compared between Ang II and the atorvastatin treatment group (Student's t-test).

reagent according to the manufacturer's protocol. Afterwards, the first strand cDNA was synthesized using the ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan). The mRNA quantification was performed using the SYBR green system. The sequences of the primers are listed in **Table 1**. The gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to the housekeeping gene Gapdh as the endogenous reference.

Western blot analysis

For the quantitative analysis of the proteins, myocardial cells were seeded in 6-well plates at a density of 30 × 10^4 cells per well. Then cells were treated as previously described for 48 h. Subsequently, a Western blot experiment was performed to determine several protein levels. The cells were dissociated in a RIPA Lysis Buffer, then the solution was centrifuged at 4°C at 10,000 × g for 10 min. The BCA (bicinchoninic acid) protein assay kit (Beyotime, China) was used to detect the total protein concentration. Subsequently, denatured proteins (20 μg) were separated by SDS-PAGE electrophoresis and then were transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk and incubated with antibodies against rat ANP, BNP, β-MHC, BCL-2, BAX, Caspase-3, p38, p-p38, JNK, p-JNK, and calcineurin (1:400) at 4°C overnight. Then the membrane was incu-

Table 2. The cell surfaces of the cardiomyocytes after treatment with Ang II or pretreatment with atorvastatin

CON	Ang II	Atorvastatin + Ang II
901.1 ± 107.8	1904.2 ± 255.6*	1202.6 ± 255.7#

*P < 0.05 compared with control group, #P < 0.05 compared between Ang II alone and combinate with atorvastatin group (Student's t-test).

bated with HRP-conjugated peroxidase-linked secondary antibody (1:5000) for 2 h at room temperature after washing. Subsequently, the bands were visualized with BeyoECL Plus. The expression was normalized with housekeeping gene expression gapdh (Beyotime, China). The bands were quantified using the Image J software.

Measurement of oxidative stress

To assay the level of oxidative stress, myocardial cells were seeded in 6-well plates at a density of 30 × 10^4 cells per well and were treated as previously described for 48 h. Then they were dissociated in a RIPA lysis buffer. Oxidative stress was determined by malondialdehyde (MDA) levels, superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activities according to the detection kits' instructions (Jiancheng Biotech, China). The absorbance was measured via a microplate reader (Themo Multiscan MK3, USA).

The ROS level was detected by 2', 7'-dichlorofluoresce in diacetate (DCFH-DA) (Beyotime, China). Briefly, cells were seeded in 12-well plates at a density of 10 × 10^4 cells per well and treated as previously described. All the procedures followed the manufacturers' directions. For the quantification of the ROS level, flow cytometry was conducted utilizing Cyan-LX (Dako Cytomation). The mean fluorescence was determined by counting the 10,000 events.

TUNEL staining

Cells were seeded in 6-well plates at a primary density of 20 × 10^4 cells per well. Drug exposure was performed as previously described. The TUNEL Staining was performed according to the instructions of the detection kits (Beyotime, China). The fluorescence intensity was quantified using the Image J software.

Measurement of intracellular Ca^{2+} concentration

To determine the concentration of Ca^{2+}, the myocardial cells were loaded with Fluo-3/AM according to the detection kits' instructions (Beyotime, China). For the quantitation of the Ca^{2+} level, flow cytometry was conducted utilizing Cyan-LX (Dako Cytomation). The mean fluorescence was determined by counting 10,000 events.

Statistics

All data were obtained from at least 3 individual experiments. All data were expressed as the mean ± standard error of the mean (SEM). The statistical analysis was performed utilizing GraphPad Prism 5 (GraphPad Software, La Jolla, CA). All data were analyzed using a one-way analysis of variance (ANOVA), with a Bonferroni correction for multiple testing. The values were considered statistically significant when (*P, #P < 0.05, **P < 0.01 and ***P < 0.001).

Results

Atorvastatin attenuated cardiac hypertrophy induced by Ang II in vitro

As shown in **Figure 1A**, the myocardial cell morphology changed much after exposure to Ang II for 48 h, accompanied by a significantly increased cell surface area, compared to the control group. Additionally, the increased size of the cell surface area induced by Ang II was inhibited by pretreatment with atorvastatin at the doses of 50 μM (**Table 2**). Moreover, there were no significant changes in cell viability among the three groups (**Figure 1B**). Thus, we performed three treatment groups to continue our study, one was the control group, and the other two were groups treated with Ang II or Ang II combined with atorvastatin.

Subsequently, three crucial markers of cardiac hypertrophy, namely ANP, BNP, and β-MHC were determined. As shown in **Figure 1C**, the mRNA levels of *ANP*, *BNP* and β-MHC were increased 2.59, 3.11 and 2.21-fold by the Ang II exposure, respectively, which was reversed by

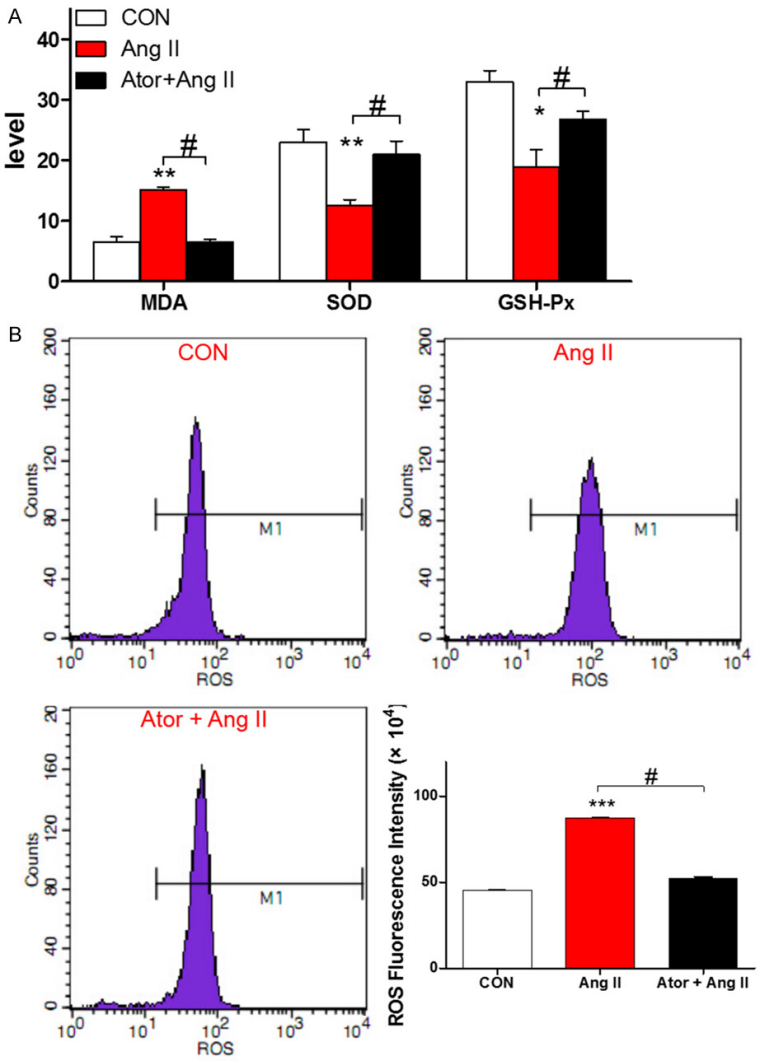

Figure 2. Atorvastatin attenuated Ang II-induced oxidative stress. A. The effects of Ang II and Atorvastatin on Myocardial Cells MDA level (nmol/mg pro), SOD activity (U/mg pro) and GSH-Px (U/mg pro). B. Quantitative analysis of the intracellular ROS generation in myocardial cells by flow cytometry; Fluorescent intensity was calculated by multiplying the number of events by the mean of the fluorescence intensity value. Data were expressed as means ± SEM. n = 3. *$P < 0.05$, **$P < 0.01$ compared with control group, #$P < 0.05$ compared between Ang II and atorvastatin treatment group (Student's t-test).

the atorvastatin treatment (**Figure 1C**). Furthermore, the protein levels of ANP, BNP, and β-MHC, determined by Western blot, were also increased by Ang II exposure, respectively, which were also prevented by atorvastatin pretreatment (**Figure 1D**).

Atorvastatin reduced ang II-induced oxidative stress

As shown in **Figure 2A**, we evaluated the MDA, SOD, GSH-Px, and ROS levels of the cardiac hypertrophy cell model. Obviously, there was an increase in the MDA levels and a decrease in the SOD and GSH-Px activities in the Ang II induced group, but atorvastatin pretreatment was found to reduce the MDA levels and enhance the SOD and GSH-Px activities. A quantitative measurement of ROS generation was conducted by flow cytometry. As shown in **Figure 2B**, the ROS generation in the cardiac hypertrophy cell model was remarkably elevated when treated with Ang II but reduced in the atorvastatin pretreatment group.

Atorvastatin inhibited cardiomyocyte apoptosis

The protein levels of Bax and caspase-3 were increased 1.69 and 1.79-fold by Ang II exposure, respectively, but reversed by atorvastatin pretreatment. However, BCL-2 was down-regulated after the Ang II exposure, whereas atorvastatin reversed this effect (**Figure 3A**). To further confirm cell apoptosis, TUNEL staining was performed. Data showed that atorvastatin pretreatment significantly suppressed the increase of the apoptotic myocardial cells (**Figure 3B**).

Atorvastatin inhibited the activation of phospho-p38 induced by Ang II

The MAPK signaling pathway-related proteins were detected by Western blot. As shown in **Figure 4**, the data showed that the phosphor-p38 and p-JNK MAPK proteins in the myocardial cells cardiac hypertrophy model were significantly increased 1.97 and 1.82-fold by the Ang II exposure, respectively, which was reduced by atorvastatin pretreatment. *Effects of atorvastatin on Ang II-induced increment of [Ca^{2+}]*

A quantitative measurement of Fluo-3/AM fluorescence intensity was conducted by flow

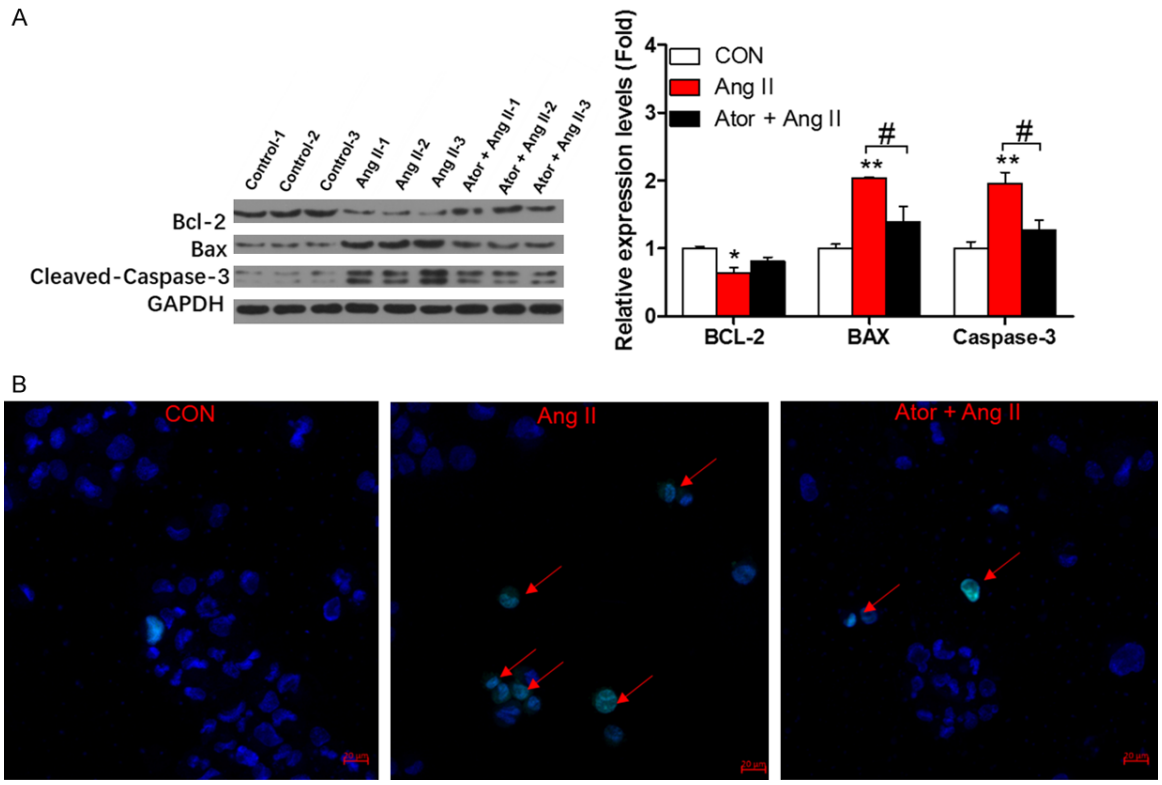

Figure 3. Atorvastatin attenuated cell apoptosis in cardiomyocytes. After treatment with Ang II alone or combined with Atorvastatin for 48 h, Western blot analysis of BCL-2, BAX and Caspase-3 in cardiomyocytes and relative expression levels of BCL-2, BAX and Caspase-3 in cardiomyocytes (A). Cell apoptosis was viewed by the TUNEL staining experiment (B). Data were expressed as the mean ± SEM. n = 3. **P < 0.05*, ***P < 0.01*, ****P < 0.001* compared with control group, *#P < 0.05* compared between Ang II alone and combinate with atorvastatin group (Student's t-test).

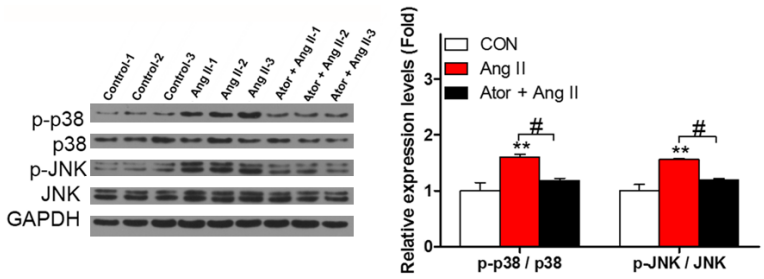

Figure 4. Atorvastatin activated the p38 MAPK signaling pathway. After treatment with Ang II alone or combined with Atorvastatin for 48 h, Western blot analysis of p-p38, p38, p-JNK, and JNK in cardiomyocytes cells and Relative expression levels of p-p38/p38, and p-JNK/ JNK in cardiomyocytes cells. Data were expressed as the mean ± SEM. n = 3. **P < 0.05*, ***P < 0.01*, ****P < 0.001* compared with control group, *#p < 0.05* compared between Ang II alone and combined with the atorvastatin group (Student's t-test).

cytometry. As shown in **Figure 5A**, the rested Ca^{2+} in the myocardial cells cardiac hypertrophy model was sharply enhanced when treated with Ang II, and then it resumed after atorvastatin pretreatment.

Additionally, the calcineurin expression was detected by Western blot. As shown in **Figure 5B**, in comparison with the control group, Ang II induced the highest level of calcineurin, whereas atorvastatin resumed the calcineurin protein level.

Discussion

To estimate the anti-hypertrophic effects of atorvastatin *in vitro*, the myocardial cells were used to establish a cardiac hypertrophy model. The results showed us that the cardiomyocytes' morphology changed much after exposure to Ang II, with the cell surface area significantly increased (**Figure 1A**). However, when pretreated with atorvastatin at a dose of 50 μM, the increased-cell surface area induced by the Ang II was inhibited (**Table 2**). These data suggest that atorvastatin has an anti-hypertrophic ability. Moreover, we did not observe any distinct changes in cell viability (**Figure 1B**). These

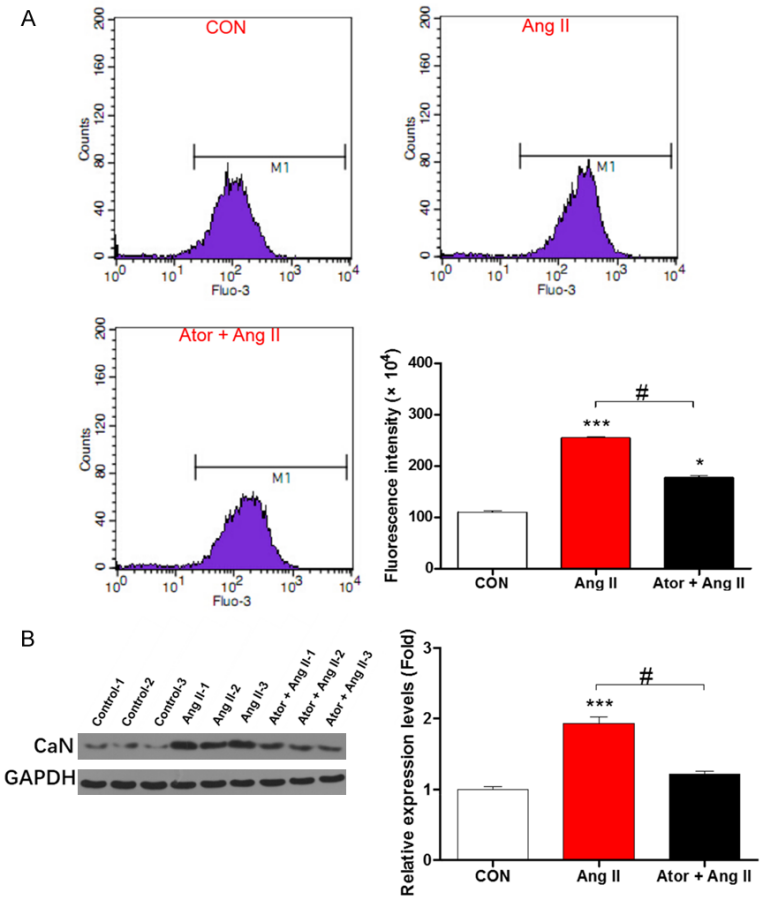

Figure 5. Effects of Atorvastatin on Ang II-induced increment of [Ca^{2+}]. After treatment with Ang II alone or combined with Atorvastatin for 48 h, quantitative analysis of the Fluo-3/AM fluorescence intensity in cardiomyocytes was conducted by flow cytometry (A). The calcineurin protein level was measured by Western blot (B). Data were expressed as the mean ± SEM. n = 3. **P < 0.05*, ***P < 0.01*, ****P < 0.001* compared with control group, #P < 0.05 compared between Ang II alone and combinate with Atorvastatin group (Student's t-test).

results suggest that atorvastatin may reduce cardiac hypertrophy at a safe dose.

To verify our hypothesis, we further determined three crucial markers of cardiac hypertrophy, namely ANP, BNP and β-MHC. The results showed us that the mRNA and the protein levels of *ANP*, *BNP*, and *β-MHC* were increased by Ang II exposure, but they were reversed by the atorvastatin treatment (**Figure 1C**, **1D**). These results indicated to us that the Ang II exposure effects were partly reversed by atorvastatin *in vitro*.

Cardiac hypertrophy is a critical phenotype component in heart failure. In the past few years, accumulating evidence has demonstrated that oxidative stress plays an important role in the pathogenesis of myocardial hypertrophy either in response to chronic pressure overload or neurohumoral stimuli [22]. Oxidative stress has been one of the dominant contributing factors that initiates apoptosis in cardiomyocytes [23]. Therefore, we detected the effect of atorvastatin on the Ang II-induced increase of oxidative stress. As shown in **Figure 2**, we evaluated the oxidative stress of the cardiac hypertrophy cell model. In the cardiac hypertrophy cell model, oxidative stress was remarkably elevated when treated with Ang II, but it was reduced in the atorvastatin pretreatment group. It has been demonstrated that the activation of Ang II type1 receptor (AT 1 R) played an important role in the development of cardiac hypertrophy, which is a reliable predictor of heart failure [24]. ROS has been proved to cause cardiac apoptosis and activate several maladaptive cascades, in turn leading to further cardiomyocyte dysfunction. The increase of ROS generation via the AT 1 receptor was the dominant factor of the negative effect of Ang II [25, 26]. When treated with Ang II, cardiomyocytes and cardiomyocytes would generate much of the ROS in cells [27]. Consistently, our findings demonstrated that atorvastatin sharply decreased the Ang II-induced increase of ROS and oxidative stress in cardiomyocytes. These data showed us that atorvastatin may be good for Ang II-induced cardiac hypertrophy.

Studies have demonstrated that cardiomyocyte apoptosis is involved in cardiac hypertrophy [28]. Thus, we determined several proteins associated with the apoptosis pathway. As shown in **Figure 3**, the data showed us that atorvastatin pretreatment significantly suppressed the increase of apoptosis in cardiomy-

ocytes. These results revealed that the Ang II-induced apoptosis was reversed by atorvastatin. It is well understood that Ang II can induce myocardial cells apoptosis due to increased oxidative stress [29]. Apoptotic cell death is a pivotal trigger for the development of Ang II-induced cardiomyopathy [30]. Our results showed that atorvastatin can reverse Ang II-induced oxidative stress, so cell apoptosis can be inhibited by atorvastatin. These well-corelated results indicate that atorvastatin pre-treatment could reduce myocardial cells apoptosis b modulating Bax and caspase-3 and by increasing the BCL-2 level. Therefore, we can conclude and suggest that pre-treatment with atorvastatin provides good protection against the Ang II-mediated pro-apoptotic signaling pathway.

Multiple studies have confirmed that Ang II elevates p38 MAPK activity [27, 31]. Thus, a Western blot assay was performed to determine the proteins involved in the MAPK pathways. We observed a significant increase in the MAPK signal pathway-related proteins after exposure to Ang II, but they were reduced by a pretreatment with atorvastatin (**Figure 4**). These results demonstrate that atorvastatin could inhibit Ang II-induced p38 MAPK activity. Investigations have reported that mitochondrial ROS are the main factors in lipid peroxidation, cell apoptosis, and p38 MAPK activation in response to Ang II in the rat myocardium [32]. The MAPK pathway was also confirmed to be a well related bio-signal with cardiac hypertrophy [33]. This was also well corelated with our previous results. Our findings showed that Ang II significantly increased intracellular oxidative stress and cell apoptosis markers along with an activation of the p38 MAPK signaling pathway. Interestingly, atorvastatin can reverse these intracellular responses efficiently, which we will investigate further.

It is understood that intracellular calcium plays a crucial role in the development of cardiac hypertrophy. Therefore, we determined the concentration of Ca^{2+} utilizing a Fluo-3/AM fluorescent probe. We observed a significant increase of the resting Ca^{2+} in our myocardial cell cardiac hypertrophy model (**Figure 5A**). Several signaling pathways have been reported to be a mediator of cardiac hypertrophy [34, 35]. Among them, some researchers revealed that Ca^{2+} was related to serine/threonine protein-phosphatase calcineurin and was a critical pro-hypertrophic signaling molecule in the myocardium [36, 37]. The activation of calcineurin in cardiac cells is sufficient to induce cardiac hypertrophy [34, 38-40]. Therefore, we detected the calcineurin protein level utilizing Western blot. Our studies revealed that Ang II induced the highest level of calcineurin but the level was reduced again by the atorvastatin (**Figure 5B**). These results indicated that atorvastatin could suppress the increase of calcineurin with the Ang II treatment, indicating the anti-hypertrophic mechanism of atorvastatin [41].

There are limitations to this study. Importantly, we did not evaluate other underlying mechanisms, which could be related to heart failure, such as the anti-inflammatory response, Rho kinase activity, autophagy, or Cyclin D1. Cardiac hypertrophy might be a result that corelates with many signaling pathways. Additionally, further studies should be performed in a clinical setting.

Taking into account the results, we demonstrated that the oxidative stress, cell apoptosis in the myocardial cell cardiac hypertrophy model. The p38 MAPK signal pathway was activated, the Ca^{2+} concentration changes were determined. Our findings demonstrated that atorvastatin possesses the capability of attenuating cardiac hypertrophy induced by Ang II. These findings demonstrate a therapeutic potential for atorvastatin in myocardial hypertrophy.

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Disclosure of conflict of interest

None.

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