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Experimental study on the role and mechanism of Allicin in ventricular remodeling through PPARα and PPARγ signaling pathways

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Abstract

The paper investigated the effects of Allicin on the changes of activities of PPARα and PPARγ signaling pathways, and the relations with the subsequent myocardial cell hypertrophy, apoptosis, myocardial interstitial remodeling, RAAS activation and heart failure.

The heart obtained from the 1-day-old Wistar suckling rats was isolated and cultured in an ordinary medium. After adding AngII and AngII+ of different concentrations respectively, the conditions of cardiomyocyte hypertrophy, apoptosis and interstitial remodeling were observed.

AngII activated the re-expression of β-MHC in cardiomyocytes, which increased the area of cardiomyocytes, indicating that myocardial hypertrophy had occurred, and the model of cardiomyocyte hypertrophy was successfully established. Compared with the control group, after being treated with different concentrations of Allicin for 24 h, the AngII-induced cardiomyocyte hypertrophy was significantly reversed, α-MHC expression increased, β-MHC expression decreased, and α/β-MHC ratio obviously increased. Allicin inhibited AngII-induced cardiomyocyte apoptosis, decreased Fas/FasL protein expression, increased Bcl-2 protein expression, and decreased Bax protein expression. The Bcl-2/Bax ratio increased significantly.

PPARα and PPARγ signaling pathways are involved in the inhibition of cardiomyocyte hypertrophy. Allicin-activated PPARα and PPARγ pathways can reverse cardiomyocyte hypertrophy, inhibit cardiomyocyte apoptosis, and alter expressions of Bcl-2/Bax and Fas/Fas-L in apoptosis-related gene.

Keywords: Allicin; PPARα; PPARγ signaling pathway; ventricular remodeling.

Practical Application: Allicin in ventricular remodeling through PPARα and PPARγ signaling pathways.

1 Introduction

Recent studies have shown that the occurrence and development of heart failure is related with ventricular remodeling (Takata et al., 2017). Ventricular remodeling is a change in the structure, function, and phenotype of cardiomyocytes caused by a series of complex molecular and cellular mechanisms. However, little is known about the complex adaptive and non-adaptive molecular regulatory mechanisms. Further discussion of the molecular mechanism of central remodeling in the heart failure process will be of great significance for clinical prevention and treatment of heart failure (Sun et al., 2016; Ruiz-Canela et al., 2016). Peroxisome proliferator activated receptors (PPARs) are a class of ligand-activated nuclear transcription factors and are members of the nuclear receptor superfamily (Le Menn & Neels, 2018). PPARs, with a variety of biological effects, can promote adipocyte differentiation and lipogenesis, enhance the body's sensitivity to insulin, regulate sugar balance in the body, inhibit the formation of inflammatory factors and inflammation, and affect tumor growth. In recent years, it was also found to produce a protective effect on blood vessels (Warden et al., 2016; Chehaibi et al., 2017; Liu et al., 2018).

Studies have shown that PPARα activity is inhibited during cardiac hypertrophy, leading to disturbances in myocardial lipids and energy metabolism. The present study aims to elucidate the changes and regularity of PPARs activity during cardiomyocyte hypertrophy, apoptosis and myocardial interstitial remodeling in ventricular remodeling, and to explore the mechanism of Allicin changing PPARs activity and cardiomyocyte hypertrophy, apoptosis, and myocardium, as well as the relationship with interstitial remodeling, RAAS activation, and heart failure.

2 Methods

2.1 Culture, identification and grouping of primary cardiomyocytes of neonatal rat

Culture of primary neonatal rat cardiomyocytes

One-day-old Wistar suckling rats were obtained. The heart was removed, the big blood vessels and atrium at the bottom of the heart were cut off, the heart tissue was cut into a meat-like

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shape in a petri dish. The tissue was gently washed. The broken tissue was moved into a 10 ml glass centrifuge tube using a straw, and an equal volume of 0.16% trypsin and 0.06% collagenase were added. The tissue was gently blown and pat, then the centrifuge tube was placed into a 37 °C water bath until the tissue block disappeared or the supernatant was clear. The supernatant was added to the serum-containing medium to terminate the digestion. The 100-mesh steel grid was placed on a small beaker, and the digested product was filtered. The filtrate was centrifuged at 1000 rpm for 10 min, the supernatant was discarded, and the precipitate was fully resuspended with complete medium (M199 + 20% FBS + double antibody). The tissue was put in an incubator with 5% CO_2 at 37 °C for 90 min. The cell suspension was inoculated into a culture flask or culture plate and incubated at 37 °C in a 5% CO_2 incubator. The BrdU at a final concentration of 0.01 mmol/L was added 2 days before the culture to inhibit fibroblast growth. The study was approved by our hospital.

Identification of cardiomyocytes

Cardiomyocytes were seeded in 6-well plates with sterile slides, cultured in an incubator at 37 °C with 5% CO_2 for 48 h. Slides with cardiomyocytes were removed, rinsed once with PBS, and fixed with 4% paraformaldehyde for 30 min. The slides were rinsed with PBS, added with 1:20 diluted normal serum blocking solution, and placed under room temperature for 10 min. The I antibody was added, and incubated at 4 °C overnight in a wet box. The slides were rinsed with PBS, added dropwise with 1:100 dilution of biotinylated goat anti-mouse IgM II antibody, placed under 37 °C for 30 min. The slides were rinsed with PBS, added dropwise with 1:100 diluted SABC-Cy3, placed under 37 °C for 30 min. The slides were rinsed in PBS, mounted and observed with a fluorescence microscope.

Experimental grouping

Cardiomyocytes were seeded in T-25 flasks at a density of 1×10^6 cells/bottle, and cultured at 37 °C with 5% CO_2 for 48 h, and then the medium was changed with serum-free medium for 24 h synchronization. According to the pretreatment method, the rats were divided into the control group; the model group, in which the final concentration of AngII is 10^{-7} mol/L and the stimulation continued for 24 h to establish the cardiomyocyte hypertrophy model; Fenofibrate group, (10 μmol/L, AngII the final stimulation concentration is 10^{-7} mol/L); Allicin group (20 μmol/L, AngII final stimulation concentration is 10^{-7} mol/L), Allicin + Fenofibrate group (20 μmol/L Allicin + 10 μmol/L Fenofibrate, AngII final stimulation concentration is 10^{-7} mol/L). 24 h before AngII stimulation, different concentrations of Allicin was administered, and 48 h after stimulation, the cardiomyocytes were rinsed with PBS to collect cells for index detection.

2.2 Determination of myocardial cell area

The cardiomyocytes from the administered groups were obtained and digested with 0.25% trypsin to complete detachment, and the cells were fully dispersed by repeated gentle blowing. After standing for about 30 min, the Leica image processing system was used for 400 × imaging. In each group, 7 views were randomly selected from the three experiments, and 100 cell areas were measured.

2.3 Transmission electron microscope observation

The culture flask with cardiomyocytes were obtained, the culture solution was aspirated, the serum and non-cellular impurities were rinsed with 37 °C PBS. The 0.29% trypsin was added to digest the cells, the cells were observed under an inverted microscope. The medium containing 10% fetal bovine serum was added to terminate the digestion to make into cell suspension. The cells were centrifuged at 1000 rpm for 10 min to be made into cell pellet. The cells were added with 4% glutaraldehyde (pH7.04) to fix at 4 °C for 8-12 h, then fixed with 2% citric acid for 2 h. The cells were dehydrated by gradient alcohol, embedded with epoxy resin, cut into ultra-thin (6000A0) sections, observed under a transmission electron microscopy, photographed, and recorded.

2.4 Annexin V-FITC/PI double labeling method to detect myocardial apoptosis

After incubating the cells in the 6-well plate for 24 h, the supernatant in the culture flask was aspirated into a centrifuge tube, and the cells were collected and digested with 0.25% trypsin without EDTA. The cells were centrifuged at 2000 rpm for 5 min, the supernatant was discarded, and the cells were washed twice with PBS (centrifugation at 2000 rpm for 5 min). The concentration of the cells to be tested was adjusted to 5×10^5-1×10^6 cells/ml. A total of 1 ml of the cells was obtained, and centrifuged at 1000 rpm for 10 min at 4 °C. The supernatant was discarded. The cells were added with 1 ml cold PBS, and gently agitated to suspend the cells. The cells were centrifuged at 1000 rpm for 10 min at 4 °C, and the supernatant was discarded. The above steps were repeated twice. The cells were resuspended in 200 μL Binding Buffer. A total of 10 μL of Annexin V-FIFC was added, mixed gently, and allowed to react at room temperature for 1 min. The apoptotic rate was measured by adding 300 μL of Binding Buffer and 5 μL of PI.

2.5 Western blot detecting the expressions of various proteins

The cells of each group were collected and washed twice with PBS. A total of 400 μL of cell lysate was added to each vial, then with 40 μL of 10 mmol/L PMSF. The vials were gently agitated, then placed on ice for 10 min to lyse the cells evenly. The cells were repeatedly aspirated with a sterile syringe. Following this, the lysed product was added to an EP tube, and the EP tube was ice-bathed for 30 min, centrifuged at 12000 g for 15 min. The supernatant was transferred to a new EP tube, and the protein concentration was quantified by a protein standard BC method. Then, every 100 μL of each tube was added with 20 μL of protein 6×Buffer and boiled for 5 min. The cells were stored at -80 °C for later use. The above samples were taken, and the proteins were separated by 12% SDS-PAGE electrophoresis, and the separated protein bands were transferred to the PVDF membrane by wet method. The membrane was blocked at room temperature for 1 h, then incubated with the primary antibody at 4 °C overnight. The cells were washed three times with PBST,

incubated with the secondary antibody (1:1000) for 1 h. The cells were washed three times with PBST. Color development and fixation were conducted by chemiluminescence. The expression of each of the above proteins was determined.

3 Results

3.1 Myocyte culture and identification

After 24 h of cell culture, the adherent growth of the cells was observed under an inverted microscope. It was columnar, fusiform, or long-polygonal. Some cells showed autonomic contraction. After 48 h, the area of adherent cells increased, and the pseudopods were extended and arranged radially. The pulsatile cells increased significantly, clustered, and the rhythm of the autonomic contraction became consistent. Immunofluorescence results showed that more than 90% of the cells expressed α-striated muscle-actin, which exhibited a filament bundle distribution, suggesting that the cultured cells were cardiomyocytes, and the purity could meet the requirements of subsequent experiments, as shown in Figure 1.

3.2 Measurement of myocardial cell surface area

The results showed that AngII induced cardiomyocyte hypertrophy and the surface area was significantly increased compared with that in the control group. The treatment of fenofibrate and Allicin for 24 h had a certain effect on cardiomyocytes, but the combination of the two drugs significantly reversed the hypertrophy of cardiomyocytes. Allicin may participate in the inhibition of cardiomyocyte hypertrophy through the activation of PPARα and PPARγ signaling pathways, possibly like fenofibrate. The effect of the two drugs on myocardial cell area is significantly additive, as shown in Figure 2.

3.3 Transmission electron microscopic observation of myocardial cells

It can be seen that the myocardial cells of the Control group are well-developed, free ribosomes and mitochondria are abundant, and the sarcomere is obvious. The adjacent cardiomyocytes are connected by intermediate connections, bridge particles and gaps. Mitochondria are mostly distributed in the perinuclear nucleus or arranged between the filaments, which are plate-like and densely arranged. The number of myocardial nuclei is 1-2, the nucleoli are clear, and the chromatin is evenly distributed. The Model group showed apoptotic cell characteristics: cell hypertrophy, chromatin condensation, aggregation under the nuclear membrane into plate-like, occasionally apoptotic bodies. The cardiomyocytes of each drug-treated group were hypertrophied, and the mitochondria were somewhat blurred, but the intact morphology of the cells was maintained, as shown in Figure 3.

3.4 Flow cytometry of apoptosis in cardiomyocytes

After staining with Annexin V/FITC and double staining with PI, the flow cytometry showed that the apoptosis rate was significantly increased after AngII induction, but decreased after pretreatment with drug. The combination of drugs exhibited more obvious effect, as shown in Figure 4.

3.5 Effect of Allicin on various proteins

The results showed that both Fenofibrate and Allicin could increase the expressions of -MHC, PPARα, PPARγ and Bcl-2 to certain extents, and both Fenofibrate and Allicin could reduce the expressions of -MHC, Bax, Fas, and Fax-L. The effect of the combination of the two drugs is more significant, suggesting that Allicin is similar to Fenofibrate as an activator of PPARα and PPARγ. The combination of the two drugs shows a significant additive effect, as shown in Figure 5.

4 Discussion

Bcl-2 family genes are divided into two categories: The first is the anti-apoptotic protein, mainly including Bcl-2, Bcl-xL, and Bcl-w, etc. The second is the pro-apoptotic protein, mainly including Bax, Bak, and Bok, etc. Overexpression of Bcl-2 inhibits a variety of apoptosis induced by many factors, while transfection with antisense Bcl-2 gene induces apoptosis in a variety of cells.

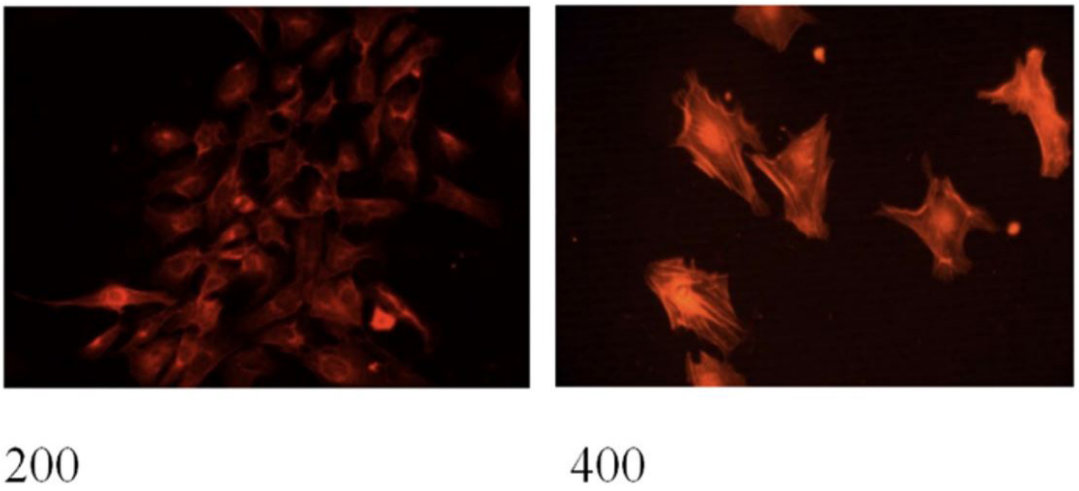

Figure 1. Cultured cardiomyocytes α-striated muscle actin of neonatal rats showed positive immunofluorescence staining.

Bax is the most widely studied pro-apoptotic protein in the Bcl-2 family. The Bcl-2/Bax ratio is important for determining whether cells enter apoptotic state (Rahimi et al., 2013).

Recently, it is found that the Bcl-2 family is widely distributed in the myocardium, and the role of the Bcl-2 family in myocardial injury has been clarified in various studies, demonstrating that Bcl-2 has an important influence on myocardial apoptosis in ventricular remodeling (Zhang et al., 2016). This present study found that, while AngII induced cardiomyocyte apoptosis, the expression of Bcl-2 protein in the proto-oncogene Bcl-2 family was decreased, and the expression of Bax increased. This indicates that the Bcl-2 family is involved in the AngII-mediated cardiomyocyte apoptosis. The addition of Fenofibrate and Allicin inhibited the apoptosis of cardiomyocytes and reversed the change of Bcl-2/Bax ratio, suggesting that Allicin is the activator of PPARα and PPARγ, like Fenofibrate, and the PPARα and PPARγ signaling pathways can be involved in the regulation of Bcl-2 family expressions.

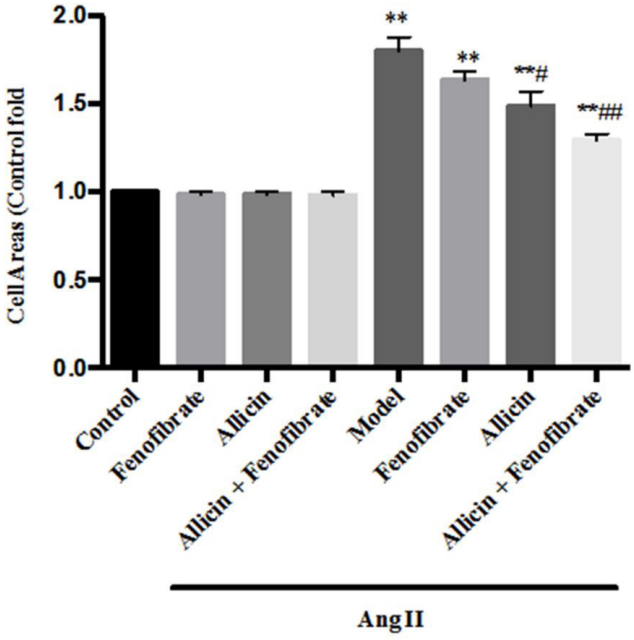

Figure 2. The Effect of Allicin on cell surface area. (**P<0.01 vs Control ##P<0.01 vs Model #P<0.05 vs Model).

Fas is also widely expressed in various tissues and is the most studied member of the TNF family. It is now known that Fas binds to the ligand Fas-L to form a receptor trimer, which in turn forms a death signal complex (DISC). The Fas C-terminal domain (DeathDomain, DD) binds to the homologous DD of the C-terminus of the DISC protein, called FADD, and activates caspases-8 with this homologous domain at the N-terminus, thereby initiating apoptosis. The Fas bypass, which regulates myocardial apoptosis signaling, also appears to follow this rule (Zhang et al., 2014; Ma et al., 2013; Li et al., 2014).

The present study found that AngII significantly elevated the expressions of Fas and Fas-L proteins in Fas family, which increased the apoptotic rate of cardiomyocytes. The pretreatment with PPARα and PPARγ ligand fenofibrate significantly reduced Fas and Fas-L, inhibited cardiomyocyte apoptosis, and Allicin played an important role, indicating that PPARα and PPARγ signaling pathways can also regulate the expression of the Fas family of apoptosis-related genes in isolated cardiomyocytes.

The effects of PPARα and PPARγ signaling pathways on apoptosis signals in cardiomyocytes of Bcl-2 and Fas may

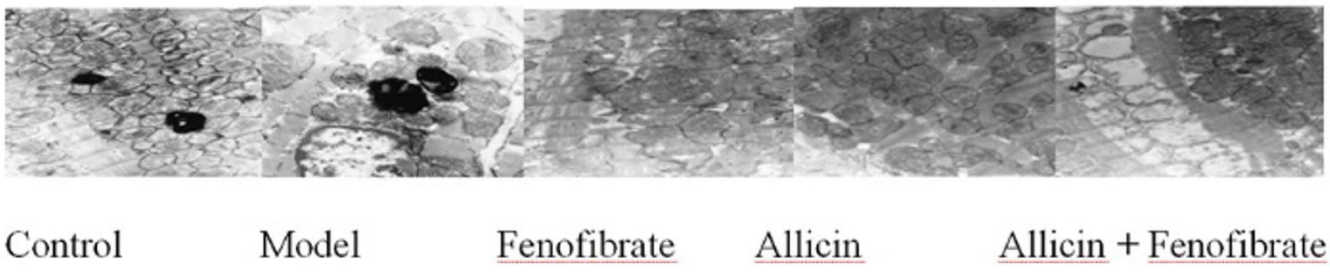

Figure 3. Effect of Allicin on myocardial cell structure.

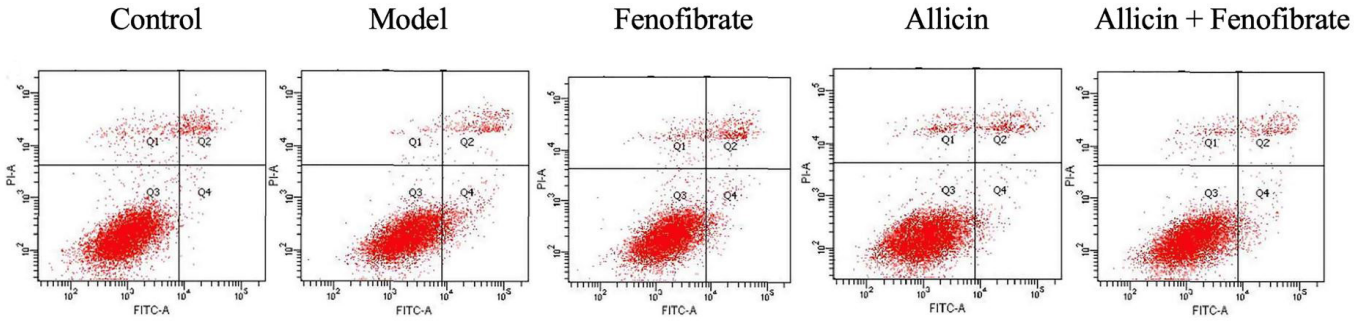

Figure 4. Effect of Allicin on cardiomyocyte apoptosis.

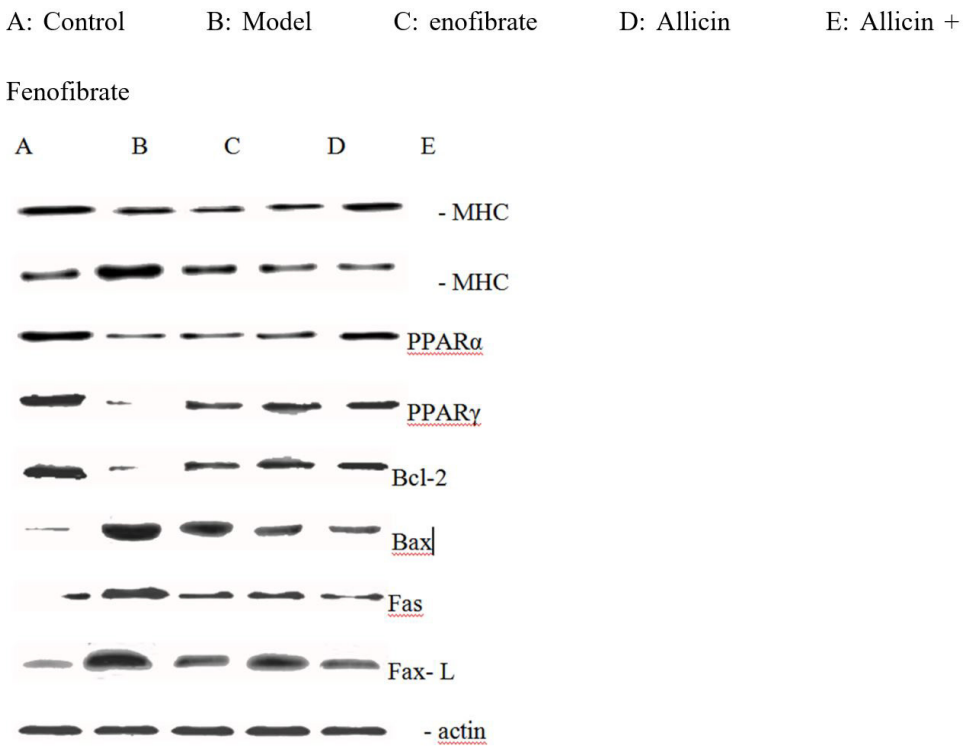

Figure 5. The effect of Allicin on various proteins.

be through that PPARs directly regulate the transcriptional expression and activation of target genes, synthesize proteins, and produce biological effects. The present study demonstrated that PPARα and PPARγ ligand fenofibrate and Allicin can significantly inhibit AngII-induced cardiomyocyte apoptosis after prolonged pretreatment and activation of PPARs signaling pathway, and regulate the apoptosis-related genes Bcl-2 and Fas family, and this may become a new entry point for the treatment of ventricular remodeling.

Ethical approval

Ethical approval has been obtained.

Conflict of interest

The corresponding author states that there is no conflict of interest.

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Author contributions

Designing research studies, Qiyun Liu, conducting experiments, Qian Fu, acquiring data, Jia Du, analyzing data, Xiaohui Liu, providing reagents, Qiyun Liu, writing the manuscript, Xiaohui Liu.

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