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miR-1 mediated AMPK pathway on cardiomyocyte apoptosis in hypertensive rats

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ABSTRACT

In recent years, it has been found that miRNA has a very close relationship with cardiovascular system diseases. Heart disease is accompanied by a change of the miRNA expression spectrum. Changing the expression of miRNA in or out of cells can cause heart diseases such as myocardial infarction, hypertrophy or arrhythmia. Mitogen-activated protein kinases (MAPKs) are important transmitters of cell surface signals to the nucleus. The family influences the biological responses of cells (e.g., proliferation, differentiation, transformation and apoptosis) by affecting the transcription and regulation of genes in animal cells. Based on the above background, the purpose of this study was to study the effect of the mir-1-mediated AMPK pathway on cardiomyocyte apoptosis in hypertensive rats. The expression level of miRNA-1 in cultured rat H9c2 cardiomyocytes was detected by real-time PCR to determine the success of the transfection. MTT method was used to detect the cell viability. Flow cytometry was used to detect the cell apoptosis, and real-time PCR and Western blot were used to detect the mRNA and protein expression of bcl-2. The results were compared with those of H9c2 cells (blank control group) and miRNA negative control fragments (negative control group). As an important kinase regulating energy homeostasis, AMPK is one of the central regulators of metabolism in eukaryotic cells and organisms, responsible for regulating cellular capacity input and output and maintaining the smooth functioning of cellular physiological activities. At the same time, AMPK is a key protein involved in a variety of signaling pathways. The results showed that the apoptosis rate of myocardial cells in the miRNA-1 group decreased (0.710 ± 0.009661)% vs (1.066667 ± 0.02603)% compared with that in the spontaneous hypertension control group ($P < 0.001$). The transfected miRNA-1mimics can up-regulate the expression of miRNA-1 in cells, inhibit the proliferation of cardiomyocytes and promote apoptosis.

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Introduction

According to the WHO's survey of major diseases that cause human diseases, hypertension is one of the main diseases that endanger human health due to its high economic burden and low quality of life. Its incidence rate and mortality rate are at the forefront of epidemic diseases. It is a risk factor for cardiovascular diseases, such as cardiovascular, cerebrovascular, kidney and organ diseases, and has aroused widespread concern. Continuous high blood pressure makes the organs such as the heart, brain, blood vessels and kidneys overloaded, resulting in structural changes and functional abnormalities. It has become one of the inducing factors of heart disease, stroke, vascular injury and renal failure. It can be seen that high blood pressure is a systemic disease (1-2).

Due to the importance of miRNA-1 research, many research teams began to study miRNA-1 and made good achievements. For example, Shen reported that the expression of muscle-specific microRNA (miRNA) miR-1 in differentiated myocytes and regenerated skeletal muscles of mice was regulated by mTOR (1-2). They found that mTOR controls MyoD-dependent transcription of miR-1 through its upstream enhancer, probably by regulating the stability of MyoD protein (3). In addition, the inhibition of histone deacetylase 4 (HDAC4) by miR-1 resulted in follistatin production and subsequent cardiomyocyte fusion downstream of mTOR and miR-1. Collective evidence strongly suggests that follistatin is a long-sought fusion factor regulated by mTOR (4-6). In conclusion, their findings first revealed the relationship between mTOR and miRNA biogenesis and identified the mTOR-miR-1-HDAC4-follistatin pathway (7-9) that regulates cardiomyocyte fusion during myoblast differentiation and skeletal muscle regeneration in vitro. Although the current research results are relatively rich, there are still shortcomings, mainly reflected in the scope of application is not wide enough.

The RASS system plays a key role in regulating blood pressure and maintaining the stability of the internal environment. Angiotensin produced in the liver is activated by renin produced in the kidney and then converted from angiotensin to angiotensin I and then to angiotensin II (Angiotensin II) produced by angiotensin-converting enzyme (ACE) in the pulmonary circulation, which then binds to the receptor ATL.

In the study of hypertension, cardiomyocyte apoptosis is closely related to hypertension, so more and more people are going to study cardiomyocyte apoptosis and

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hypertension. The balance between cell death and cell survival is a strictly controlled process, especially in terminally differentiated cells, such as cardiomyocytes (10-11). The accumulated data support the role of cardiomyocyte apoptosis in the development of various heart diseases, including the transition from hypertensive compensatory hypertrophy to heart failure (12). Jiang made a review on the research progress of myocardial cell death survival balance in patients with hypertensive heart disease (13-14). Some molecular and cellular aspects and the most relevant pathophysiological implications were introduced (15). In addition, the possibility of developing diagnostic tools and drug interventions was also studied (16-17). Because of the effectiveness of hypertension research, whether the detection of cardiomyocyte apoptosis can be applied to the study of hypertension to solve the problem of whether hypertension affects cardiomyocyte apoptosis.

RAS (renin-angiotensin-aldosterone system) candidate gene

The abnormality of the blood pressure regulation mechanism is a common disorder of regulation center in hypertension, which leads to a rise in blood pressure. Most of the angiotensin II in the body combines with its AT1 to play a biological effect. It can activate the cells of the glomerular zone of the adrenal cortex to secrete aldosterone, which plays a role in water conservation and sodium reabsorption (18). The effect of ANG-II on VSMCs can stimulate their contraction and proliferation, increase the hardness of the vascular wall, reduce diastolic function, and directly promote the formation and development of hypertension. Vascular smooth muscle cells are the main cells that constitute the vascular structure and maintain vascular tension. Hypertension causes the continuous increase of vascular tension, resulting in the proliferation and hypertrophy of vascular smooth muscle cells, migration to the membrane, and thickening of the vascular wall. This pathological change of blood vessels also causes a continuous increase in blood pressure (19-20).

ACE gene expression plays a key role in hypertension. Ace does not play a significant role in regulating human hypertension (21). In conclusion, most of the research results have proved that act gene expression level is increased, and it is more likely to suffer from essential hypertension (22). The decrease in ACE2 expression may also be an important factor in hypertension.

Natriuretic hormone candidate gene

The natriuretic peptide gene is also closely related to primary hyperemia. Natriuretic peptide includes atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and D-type natriuretic peptide (DNP); ANP and BNP are synthesized in the heart, CNP is produced by blood vessels, DNP is produced by atrial myocytes (23-24). These gene deletions or abnormal expression are also one of the multiple pathogenic factors of essential hypertension, some of which are also induced by other diseases.

Atrial natriuretic peptide

Atrial natriuretic peptide (ANP) is a vascular regulatory peptide that has the functions of diuresis, sodium excretion, vasodilation, inhibition of sympathetic nerve excitability, RASS system, and reduction of cardiac load. ANP is synthesized and secreted by cardiomyocytes (25). Atrial natriuretic peptide gene abnormality is not only related to the pathogenesis of hypertension but also closely related to a variety of diseases, such as heart disease.

This experiment aimed to study the relationship between miRNA-1 level and the expression of Bcl-2, an apoptosis regulatory gene, and to explore the role of miRNA-1 in cardiomyocyte apoptosis in hypertensive rats. The apoptosis rate of myocardial cells in the miRNA-1 group and the spontaneous hypertension control group decreased the apoptosis rate of myocardial cells in the miRNA-1 group.

Materials and Methods

Main reagents and instruments

Rat H9c2 myocardial cell line was purchased from the Shanghai cell bank of the Chinese Academy of Sciences. DMEM medium, fetal bovine serum (FBS), trypsin, transfection reagent Lipofectamine 2000, RNA extraction reagent Trizol, reverse transcriptase m-mlv, RNA enzyme inhibitor, real-time PCR kit, miRNA-1mimics and miRNA negative control fragment, bcl-2 antibody (Abcam company), β - actin antibody, fitcnnexinv apoptosis kit. Abi7500 real-time fluorescent quantitative PCR instrument, flow cytometer, AMPK inhibitor compound C; one anti-collagen I and III, one anti matrix metalloproteinase (MMP-2, MMP-9), one anti vimentin are all products of Abcam company in the United Kingdom, one anti-microtubule related protein 1 light chain 3B - II (lc3b - II), one anti AMPK and phosphorylated AMPK (p-ampk) are all products of cell signaling company in the United States, Recombinant protein 1, β - actin (β - actin, sigma company of the United States).

Cell culture and transfection

Rat H9c2 cardiomyocytes were cultured in a DMEM medium containing 10% FBS at 37 ℃ and 5% CO2. On average, every 3 days. When the cell growth density reaches about 70% fusion, change the solution to standby. After 24 hours, the cells were divided into three groups. Blank control group: H9c2 cells cultured under normal conditions; negative control group: according to the instructions of the transfection reagent Lipofectamine 2000, the randomly synthesized miRNA negative control segments were transfected; miRNA-1 group: H9c2 cells transfected with miRNA-1mimics segments. Mix miRNA-1mimics dissolved in serum-free DMEM with Lipofectamine 2000 and incubate at room temperature for 20min, then add the mixture to the cell culture medium and put it into the incubator for 48h.

Detection of miRNA-1 by real-time PCR

According to the instruction manual for RNA extraction reagent Trizol, the total RNA of cells was extracted, and the concentration and purity of total RNA were detected by ultraviolet spectrophotometer. The integrity of total RNA was detected by formaldehyde denaturation agarose gel electrophoresis. The cDNA was synthesized by reverse transcription and detected by real-time PCR with U6 as an internal reference. The PCR amplification conditions were: pre denaturation at 95 ℃ for 5min, 10s at 95 ℃, 30s at 60 ℃, 40 cycles. PCR product generation is exponentially increasing, capable of amplifying starting templates to be tested on the order of picograms (pg = 10-12) to the

microgram (μg = 10-6) level. The PCR can detect one target cell from one million cells; in the detection of viruses, the PCR has a sensitivity of up to 3 RFU (empty spot forming units); in bacteriology, the minimum detection rate is 3 bacteria. The PCR reaction is carried out with a high-temperature resistant Taq DNA polymerase once the reaction solution has been spiked, i.e. denaturation-annealing-extension reaction is carried out on the DNA amplification solution and water bath, usually in 2 to 4 The amplification reaction is usually completed in 2-4 hours. The amplification products are generally analysed by electrophoresis, not necessarily with isotopes, without radioactive contamination and easily promoted.

Cell survival test

One day before the transfection, the logarithmic growth cells were inoculated into 96 well plates, 1 × 104 cells per well. 24 hours later, miRNA-1mimics and miRNA negative control fragments were transfected. The cells without transfection were used as blank control, and the holes with only culture medium were used as zeroing holes. 48 hours later, 20 μ l of 5 g / L MTT was added to 96 well plates and incubated in a 5% CO2 incubator at 37 ℃ for 4 hours. Suck up the pore liquid, add 150 μ l DMSO, and place it on a horizontal shaking table at room temperature until the crystal is completely dissolved. The absorbance (a) of the solution was measured at 490 nm. The cell survival rate of the blank control group was 100%, and the cell survival rate of the experimental group (%) = a value of the experimental group / a value of the blank control group × 100%.

Western blot analysis of protein expression

The supernatant was taken. The protein content was determined by the BCA method. The protein was sampled at 50 g, and then transferred to nitrocellulose membrane (NC membrane) or poly (vinylidene fluoride two fluoroethylene membrane) (PVDF membrane) by twelve alkyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 5%BSA was used to block 1H on the membrane nonspecific antigen. Add one anti-collagen I (1:500), one anti-collagen III (1:100), one anti-MMP-2 (1:1000), one anti-MMP-9 (1:1000), one anti lc3b II (1:500), one anti p62 / sqstm1 (1:1000), one anti β - actin (1:5000), one anti AMPK (1:1000) and one anti p-ampk (1:1000) respectively, and incubate in the refrigerator at 4 ℃ overnight; wash with phosphate buffer (tbst) containing Tween 20 for three times, incubate at room temperature for 1 h after adding the corresponding second antibody. Using Odyssey developer to develop ImageJ software to collect and analyze the image and determine the gray value of each band.

Statistical treatment

Spss20.0 software was used to analyze the data and graphpadprism7 software was used to process the data. All the data were expressed as mean ± standard deviation (x ± s). Single-factor analysis of variance was used for comparison between groups. LSD-t-test was used for comparison between the two groups. 1 < 0.05 was statistically significant.

Results

The level of miRNA-1 in cells of each group

There was a significant difference in miRNA-1 among the three groups (F = 13.73, P < 0.05). The relative expression of miRNA-1 in miRNA-1 group was 3.46 ± 1.28, significantly higher than that in blank control group (0.66 ± 0.14) and negative control group (0.71 ± 0.16) (P < 0.01).

MTT assay for cell survival rate

The difference in cell survival rate among the three groups was statistically significant (F = 69.60, P < 0.05). The cell survival rate of the miRNA-1 group was 45.69% ± 8.13%, which was significantly lower than 97.87% ± 10.21% of the negative control group and 100% ± 8.61% of the blank control group (P < 0.01).

Real-time PCR detection of cardiomyocyte apoptosis in young spontaneously hypertensive rats

The results of myocardial apoptosis in young SHR by real-time PCR are shown in Figure 1.

According to the MTT rule, the higher the cell survival rate, the lower the apoptosis, and the lower the cell survival rate, the higher the apoptosis. The green fluorescence observed under the 400X fluorescence microscope is the apoptotic cell, red light is the myocardial tissue structure, and blue light is the DAPI staining result. The data on cardiomyocyte apoptosis rate is shown in Table 1. The changing trend of cardiomyocyte apoptosis rate in each group is shown in Figure 2.

Based on the data in Figure 2, it is clear that the data for the SHR group is lower than the data for the SHR+e group, so this conclusion is consistent with the data in

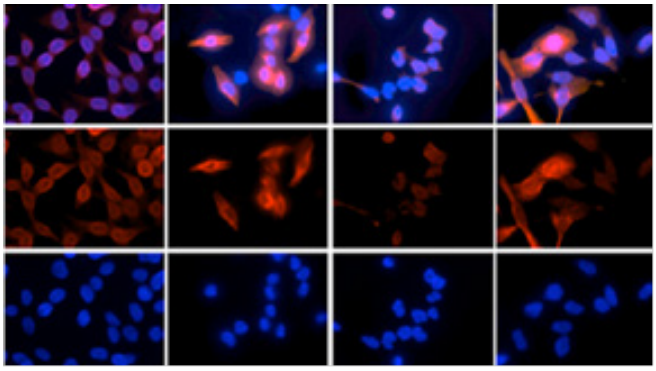

Figure 1. The myocardial apoptosis in young SHR.

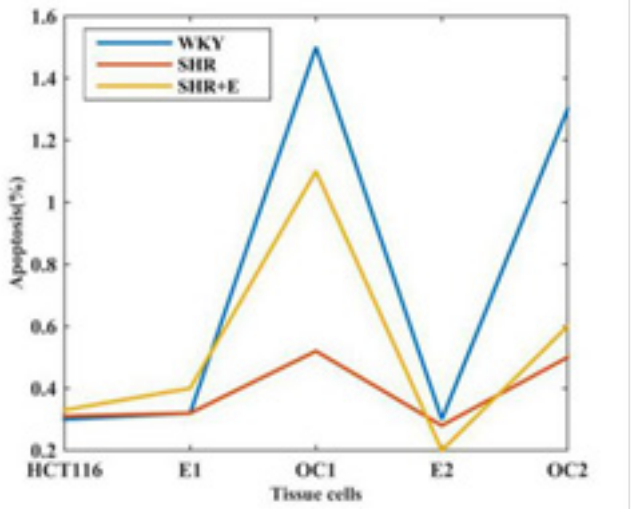

Figure 2. Trends of rat cardiomyocyte apoptosis rate in each group.

Table 1. The apoptosis rate of rat myocardial cells in each group.

Group	Sample	Apoptosis rate (%)
WKY	6	0.3167±0.01174
SHR	6	1.067±0.02603***
SHR+E	6	0.710±0.009661###

***Compared with WKY group, P < 0.001; compared with SHR group, P < 0.001, SHR-E is a monoclonal antibody inhibitor. Primarily, the monoclonal inhibitor is injected into experimental subjects to observe the rate of apoptosis in cardiomyocytes. E is for positive immune cells.

Table 1. It can be seen from the figure that the number of apoptotic cells in the SHR group is higher than that in the WKY group. The experimental data have significant difference (1.067 ± 0.02603) vs (0.3167 ± 0.01174), * * * P < 0.001. Through the AMPK pathway mediated by miR-1, the number of apoptotic cells in the SHR group is significantly lower than that in SHR + e. the experimental data have a significant difference (1.067 ± 0.02603% vs (0.710 ± 0.009661)%, ###p < 001. These results suggest that the abnormal apoptosis of cardiomyocytes has occurred in the early stage of hypertensive rats, and the apoptosis rate of SHR cardiomyocytes decreased after the AMPK pathway mediated by miR-1.

The changing trend of the optical density values of Bcl-2, Bax and Caspase-3 in the hearts of the three groups of rats is shown in Figure 3.

From the following experimental data, we can find that compared with the SHR group, the experimental data of bcl-2 expression in the WKY group has no statistical significance (P > 0.05), Bax and Caspase-3 expression significantly increased (P < 0.05); after 8 weeks of swimming, compared with SHR + e group, bcl-2 expression increased, Bax and caspases-3 expression decreased, and the experimental data has statistical significance (P < 0.05).

Detection of apoptosis by flow cytometry double staining

48 hours after transfection of miRNA-1mimics, the apoptosis rate was 15.7% ± 2.82%, significantly higher than the blank control group (3.13% ± 0.45%) and the negative control group (3.33% ± 0.70%), the difference was statistically significant (F = 54.06, P < 0.01), as shown in Figure 4.

Flow cytometry is a biological technique for the counting and sorting of tiny particles suspended in fluid. It is a technique for the quantitative analysis and sorting of single cells or other biological particles in suspension by detecting a labelled fluorescent signal at high speed and on a cell-by-cell basis and can be used for continuous multiparameter analysis of individual cells flowing through an optical or electronic detector.48 hours after transfection of miRNA-1 mics, the mRNA expression level of Bcl-2 was significantly lower than that of the blank control group (2.38 ± 0.13) and negative control group (2.39 ± 0.15) (F = 57.80, P < 0.01). There was no significant difference between the blank control group and the negative control group. The protein expression level of Bcl-2: there was no significant difference between the blank control group (0.95 ± 0.05) and the negative control group (0.94 ± 0.11). The miRNA-1 group (0.50 ± 0.10) was significantly lower than the blank control group and the negative control group (F = 23.76, P < 0.01).

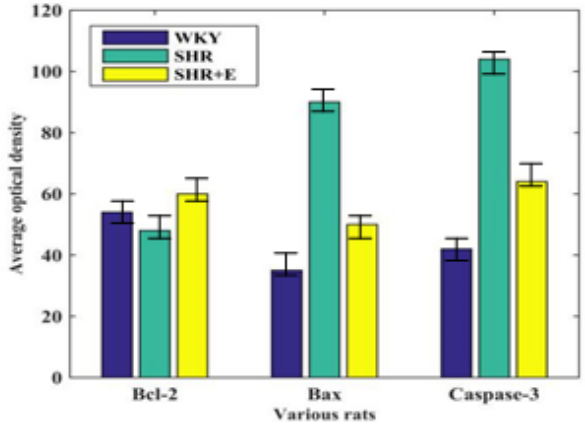

Figure 3. Average optical density values of bcl-2, bax and caspase-3 expression in the heart of rats in each group.

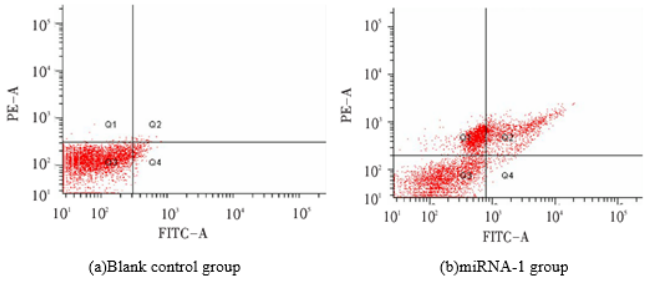

Figure 4. Flow cytometry double staining method to detect the apoptosis rate.

Discussion

Hypertension has been considered as a risk factor for heart disease, and the mechanism of heart injury has been partially explained. It has been proved that there is abnormal apoptosis in hypertensive cardiomyocytes by predecessors, but at present, no drugs can directly inhibit the abnormal apoptosis of hypertensive cardiomyocytes to prevent and alleviate the disease. This study found that miRNA plays a key role in all processes of cell development, regulating cell proliferation, differentiation, aging, death and maintaining cell self-renewal. In recent years, miRNA-1 has been a kind of microRNA related to heart disease, including miRNA-1-1 and miRNA-1-2. The length of the miRNA-1 gene is 21bp. MiRNA-1 is only expressed in the myocardium and skeletal muscle, which is considered to have myocyte specificity. Its expression is regulated by transcription factors such as serum response factor, myocyte enhancer-2 (MEF2), MyoD and muscle regulatory factors (MRFs).

Cell differentiation produces differences in cells in space and the same cell in time from its previous state. Over time, cells gradually decline in their ability to proliferate and differentiate and in their physiological functions. Cellular fibrosis refers to fibrinoid necrosis, a type of necrosis that occurs in the mesenchyme, collagen fibres and small vessel walls.

In this study, the expression of collagen I, collagen III, MMP-2 and MMP-9 in the Hg + lv-mir1 group was significantly higher than that in Hg + lv-vehicle group. The basic process is to first separate the sample by SDS-polyacrylamide (SDS-PAGE with 0.1% gelatin) electrophoresis, then

rejuvenate MMP-2 and MMP-9 in the sample in a buffer system in the presence of divalent metal ions, hydrolyse the gelatin in the gel at their respective migration sites, and finally stain the gel with Komas Brilliant Blue and then decolorize it so that a white band can appear against a blue background The experimental parameters were then obtained. The results showed that AMPK inhibitor compound C increased the degree of myocardial fibrosis and reversed the effect of miR-1 gene silencing on the degree of myocardial fibrosis. After AMPK inhibitor treatment, the expression of lc3b - II and p62 / sqstm1 increased significantly, indicating that autophagy flow is not smooth at the moment. In general, from the point of view that the reduction of myocardial fibrosis caused by miR-1 silencing can be reversed by AMPK inhibitors, this study verified that miR-1 silencing may play a role in myocardial protection through the influence of the AMPK pathway on autophagy flow.

To sum up, silencing miR-1 can activate the AMPK pathway, promote autophagy and reduce the degree of myocardial fibrosis caused by hypertension, but the relationship between miRNA, AMPK pathway and autophagy and myocardial fibrosis is not clear. In conclusion, it can be concluded that miR-1 can activate the AMPK pathway, promote autophagy and reduce the extent of myocardial fibrosis induced by hypertension. miR-1-mediated AMPK pathway can reduce apoptosis in rat cardiomyocytes caused by hypertension.

Acknowledgments
Not applicable.

Interest conflict
The authors declare that they have no conflict of interest.

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