MECHANISM OF BERBERINE ON INHIBITING MYOCARDIAL HYPERTROPHY

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ABSTRACT

Introduction*: In primary cultures of neonatal rat cardiomyocytes, we studied the effect of berberine (BBR) on phenylephrine (PE)-induced cardiomyocyte hypertrophy and the mechanisms of its regulation of miRNA-199a-5p.*

Materials and methods*: Cultured neonatal rat cardiomyocytes were divided into : Control group (no treatment), PE group (with 10 μl/ml of 10 μM PE stock), and PE + BBR group (with 10 μl/ml of 10 μM PE and 10 μg/ml BBR). The cardiomyocyte surface area was measured. The incorporation rate of 3(H)-leucine was used to measure cardiomyocyte hypertrophy. The expression of miRNA-199a-5p in cardiomyocytes was measured. The candidate target genes of miRNA-199a-5p were analyzed and the expression level of Hsp70 was detected.*

Results*: BBR inhibited PE-induced cardiomyocyte hypertrophy and significantly increased the rate of incorporation of 3(H)-leucine. miRNA-199a-5p was significantly up-regulated in PE group; the expression of miRNA-199a-5p was similar in the PE + BBR group and the control group, while the expression of Hsp70 protein was significantly increased in the PE + BBR group compared with the PE group.*

Conclusion*: miRNA-199a-5p may play an important role in cardiac hypertrophy, and BBR can inhibit PE-induced cardiomyocyte hypertrophy, which may be related to the inhibition of up-regulation of miRNA-199a-5p and increasing the level of Hsp70.*

Keywords*: berberine, phenylephrine, cardiomyocyte hypertrophy, miRNA-199a-5p, Hsp70.*

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Introduction

MicroRNA (miRNA) is a class of intracellular non-coding RNA with a size range of 18-25 bp that binds to the 3´-untranslated region of target mRNA, inhibiting the translation of proteins or regulating the stability of mRNA[(1)]. High expression of several species of miRNA is found in myocardial tissue, and studies have shown that these miRNAs play an important role in myocardial development and heart disease, and are thus potential new drug targets for the treatment of heart disease[(2)]. The aberrant expression of miR-199 in cardiac hypertrophy can reduce the injury to cardiomyocytes induced by myocardial ischemia and reperfusion, and inhibit cardiomyocyte apoptosis[(3, 4)].

Berberine (BBR) is a common isoquinoline alkaloid, an important component of the Chinese herb Coptis chinensis. Its molecular formula is C2OH18NO4, and it was initially used as a detoxifying and antibacterial drug in clinical medicine. Further studies found that BBR has a wide range of pharmacological effects on the cardiovascular system, including a positive inotropic effect on the heart[(5)], an antiarrhythmic effect[(6)], blood vessel dilation and antihypertensive effects[(7)], and properties of anti-myocardial ischemia[(8)], anti-type 2 diabetes and anti-obesity[(9-11)]. BBR has also reportedly been used for treatment of cardiovascular diseases such as heart failure[(12)], arrhythmia[(13)], and hypertension[(14)]. Due to the effects of BBR on the cardiovascular system, we speculate that BBR may play a protective role in cardiac hypertrophy.

In this study, primary cultures of neonatal rat cardiomyocytes were used to observe the effect of

BBR hydrochloride on the expression of miRNA-199a-5p protein and its target protein Hsp70 in cardiomyocyte hypertrophy, and to explore the protective mechanism of BBR on myocardial hypertrophy, thus providing experimental evidence for clinical applications.

Methods

Culture of cardiomyocytes

All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Quzhou College of Technology and adhere to national regulations for animal experimentation. SD rats, 1 to 2 days old, (provided by the Experimental Animal Center of Zhejiang Academy of Medical Sciences) were immersed in 75% alcohol for disinfection, then taken out immediately and sacrificed. The ventricular tissues were cut with scissors in a sterile fashion on a clean bench (SW-GL-2FD, Suzhou Antai Airtech Co., Ltd.), then washed in D-Hanks solution 3 times. After the atria and blood clots were stripped off, the ventricular tissues were cut into 1 mm3 pieces with scissors. A solution of 1% collagenase Type II and D-Hanks solution were mixed in a ratio of 1:4 to prepare a digestion solution. The ventricular tissue fragments were digested in this solution at 4ºC in a refrigerator and left overnight. The digestion solution was removed by pipetting, and digestion was terminated with stop solution (4 ml Dulbecco's

Modified Eagle's Medium (DMEM), containing high glucose (500 mg/l) and 10% (v/v) fetal bovine serum, added to 10 ml digestion solution), then the preparation was centrifuged at 1000 rpm for 15 min. After cell counting, the cells were seeded in 12-well plates at a density of 3–5×105/ml, 10 μl/ml prepared antibiotic solution(10,000 units of penicillin and 10,000 μg streptomycin/ml) in high-glucose DMEM was added, and the plates were incubated in a 37ºC CO_2 incubator. After 24 h of incubation, the cells were washed with phosphate-buffered saline (PBS, cell culture grade) and then the medium was replaced with high-glucose DMEM containing 10% fetal bovine serum and 0.1 mM BrdU to suppress the proliferation of the small number of non-cardiomyocytes. Incubation was continued for another 48 h, then the medium was replaced with serum-free and antibiotic-free high-glucose DMEM.

Grouping and administration

After been incubated in serum-free and antibiotic-free high-glucose DMEM for 24 h, the drugs were administered. These wells were divided into three groups, a control group (no treatment), a PE group (with 10 μM PE, 10 μl/ml), and a PE + BBR group (with 10 μM PE, 10 μl/ml and 10 μg/ml BBR). For each group, the solution was shaken slightly to mix, then incubated for 48 h in a cell incubator at 37ºC.

Determination of cardiomyocyte area

The cardiomyocytes were seeded in 12-well plates with 4 parallel holes for each group. After 48 h of treatment, the culture solution was discarded and cells were quickly rinsed twice with PBS, then the cells were observed and photographed under an inverted microscope (objective lens 20×) with five separate fields per well and 5 cells per field. Image-Pro Plus 5.0 professional image analysis software (Media Cybernetics, Bethesda, MD, USA) was used to measure individual cell area in μm2.

3(H)-leucine incorporation assay of cardiomyocytes

At 24 h after starting incubation of cardiomyocytes in serum-free culture medium, the cultures for each group received the additions mentioned above for treatment, along with 18.3 kBq 3(H)-leucine, then culturing was continued for another 24 h. The culture medium was discarded and the cells were rinsed twice with PBS. The cells were harvested on a cellulose filter film after digestion with 25 mg/l trypsin, fixed with 10% trichloroacetic acid, and the filter membrane was dried. A LS3910 liquid scintillation counter (Beckman Coulter, Shanghai) was used to measure radiation intensity.

Analysis of expression of miRNA-199a-5p by real-time PCR

The miRNA-199a-5p primer (sequence 5´-CCCACTGTTCAGACTACCTGTTC-3´) was synthesized by Invitrogen Co., Ltd. (Shanghai). The sequence for the internal standard U6 primer was 5´-TTCGTGAAGCGTTCCATATTTT-3´. At 24 h after treatment, TRIzol reagent was added to each group of cardiomyocytes. The lysate was transferred to a new tube, chloroform was added, and the mixture was shaken and centrifuged. The supernatant was transferred to a new tube and mixed with isopropyl alcohol, the supernatant was

discarded after centrifugation, and the RNA was washed with 75% ethanol prepared with DEPC-treated water. After centrifugation, the supernatant was discarded. The proper amount of DEPC-treated water was used to fully dissolve the RNA. The RNA solution was diluted, then its concentration was measured. The corresponding miRNAs were reverse-transcribed using a stem-loop primer, followed by real-time PCR assay. A double standard curve method was used to quantitate the miRNA-199a-5p concentration of each sample. Then, 500 ng RNA was reverse-transcribed according to the following reaction mixture of 10 μl: 2 µl dNTP (2.5 mM stock), 0.5 µl RT primer (10 µM), 2 µl oligo dT (10 µM), 2 µl 5× first strand buffer, 0.1 µl DTT, 0.4 µl reverse transcriptase, x µl RNA, (3-x) µl DEPC H2O. Reaction conditions were 16ºC 30 min; 42ºC 90 min; 72ºC 5 min. The reverse transcription products were diluted 50-fold and analyzed by real-time fluorescence quantitative PCR using the following system: 5 µl 2× miRcute miRNA premix (with SYBR and ROX), 0.8 µl 50× ROX reference dye, 0.2 µl forward primer, 0.2 µl reverse primer, 1 µl cDNA, 2.8 µl ddH2O. Reaction conditions were 95ºC 2 min; 94ºC 15 s; 60ºC 1 min for 40 cycles.

Western blot assay of Hsp70 expression

The cardiomyocytes in 12-well plates had 60 µl protein lysis buffer added to each well and were left for 15 min at room temperature for lysis. The supernatant was transferred using a sterile pipette tip, centrifuged at 12,000 rpm for 30 min at 4ºC, and the supernatant was collected and stored in a -20ºC freezer for future use. After measurement of protein concentration of cardiomyocytes in each group, SDS–PAGE loading sample buffer (reducing, 5×) and the diluted protein samples were mixed at a volume ratio of 1:4, then were denatured in a constant temperature (100ºC) water bath for 5 min. The denatured protein samples were added into the stacking gel at 500 ng protein per gel well for electrophoresis, with the voltage set at 80 V. After the protein reached the resolving gel, the voltage was adjusted to 100 V. After electrophoresis, proteins in the resolving gel were transferred to a PVDF membrane. Transfer conditions were constant current 200 mA for 120 min. After transfer, the membrane was placed in blocking buffer. After blocking, the membrane was incubated overnight with a 1:20,000 dilution of primary antibody at 4ºC, then incubated with 1:5000 dilution of secondary antibody at room temperature for 2 h. Bound antibodies were detected with enhanced chemiluminescence (ECL) and photographed with a Gel Imaging System (Bio-Rad), and the results were scanned in gray-scale mode for analysis.

Statistical analysis

All data are expressed as mean ± SD; t-tests were performed using SAS 9.0 statistical software (SAS Institute Inc., Cary, NC USA). A value of $p<0.05$ was considered statistically significant.

Results

Effects of BBR on cell area of hypertrophic cardiomyocytes

The cell area in the PE group was significantly greater than in the control group and PE + BBR group ($p<0.05$); there was no significant difference in cell area of the PE + BBR group and the control group (Table 1).

Group	Area (μm^2)
Control	240.41 ± 126.00
PE	615.44 ± 166.5*†
PE + BBR	283.39 ± 147.21

Table 1: Effect of BBR on area of hypertrophic cardiomyocytes ($\bar{x}\pm s$).
**Compared with control group ($p<0.05$), †compared with PE + BBR group ($p<0.05$)*

Effects of BBR on the rate of protein synthesis in cardiomyocytes

At 24 h after administration, the rate of protein synthesis (1895 ± 110 cpm) in the PE group was significantly increased compared with the control group (1175 ± 104 cpm) ($p < 0.01$); BBR inhibited the increased protein synthesis induced by PE; there was a significant difference in the rate of protein synthesis in the PE + BBR (1227 ± 102 cpm) and PE groups (1895 ± 110 cpm) ($p <0.05$).

Analysis of RNA samples from cardiomyocytes and fluorescence quantitative PCR of miRNA-199a-5p

RNA samples of cardiomyocytes from each group showed (A260/A280) values >1.9, and 28S/18S values of 1.6–1.7, confirming high concentration of the extracted RNA. The expression of miRNA-199a-5p in the three groups of cardiomyocytes after 24 h of treatment is shown

in Figure 1. miRNA-199a-5p in the PE group was up-regulated compared with the control and PE + BBR groups, while the expression of miRNA-199a-5p in the PE + BBR and control groups was similar.

Bioinformatics analysis using TargetScan software (http://www.targetscan.org) and PicTar software (pictar.mdc-berlin.de) was used to predict target genes of miRNA-199a-5p. HSPA12A, i.e. Hsp70, was a potential target gene of miRNA-199a-5p (Fig. 2).

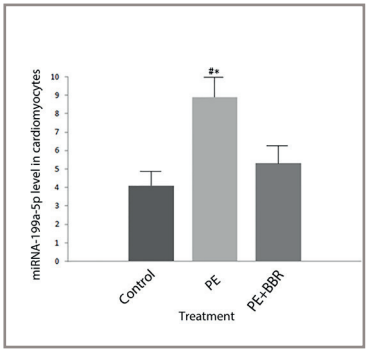

Figure 1: miRNA-199a-5p level in cardiomyocytes.
*#p<0.05 vs. control group, *p<0.05 vs. PE + BBR group, n=4*

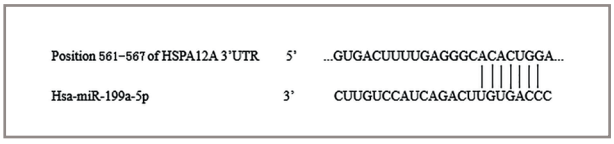

Figure 2: Potential targets of miRNA-199a-5p.

Effects of BBR on the expression of myocardial Hsp70

At 24 h after administration, expression of myocardial Hsp70 in the BBR group was 41.7 ± 0.15 times that of the control group and 11.9 ± 0.22 times of that of the PE group ($p < 0.05$, $n = 3$) (Figure 3).

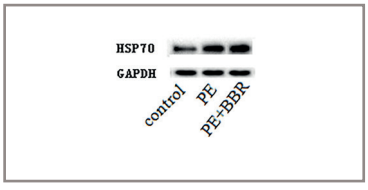

Figure 3: Effects of BBR on expression of HSP70 protein in myocardial hypertrophy (n=3).

Discussion

In recent years, research on molecular mechanisms of cardiac hypertrophy has been progressing rapidly, and a series of positive or negative regulatory signaling molecules of cardiac hypertrophy have been found, including miRNA, one of the most important signaling molecules. Previous studies showed that miRNA can regulate physiological processes such as cell division, differentiation, proliferation and apoptosis, embryonic development, energy metabolism, hormone secretion, hematopoietic function and reaction to stress, as well as pathological processes such as cancer and diabetes[15-17].

In the cardiovascular system, miRNA is involved in the development of the heart and blood vessels, as well as the occurrence of disease[18, 19]. Studies on miRNA regulatory mechanisms in pathological cardiac hypertrophy have shown that miRNA-199a-5p may play an important role in cardiac hypertrophy, and that overexpression of miRNA-199 in cardiomyocytes can promote myocardial hypertrophy at the basal level[20].

Heat shock proteins (HSPs) are a family of proteins that are newly produced or produced at increased levels by cells in response to stressful conditions, and are also known as stress proteins. HSPs are a big family with many members, and according to their molecular weights, they can be divided into subfamilies. HSPs are highly conserved evolutionarily; both prokaryotic cells and eukaryotic cells express HSPs, and HSPs in the same family have similar structure and functions. The main biological function of HSPs is to help folding, displacement, refolding and degradation of proteins, and HSPs are therefore called molecular chaperones[21]. Hsp70 is the most conserved and most studied HSP subfamily, and can be divided into a constitutive isoform and an inducible isoform.

Constitutive Hsp70 is located in the cell cytoplasm, mainly as a molecular chaperone, while inducible Hsp70 is widely present in the cytoplasm and the nucleus, and is also detected in the cell membrane. HSP expression is low in normal cells; aberrant expression of Hsp70 plays an important role in the development of the disease process: When cells are subjected to a variety of stresses, expression of inducible HSPs is increased in order to increase cell resistance to harmful stress; it is the anti-injury reaction at the cellular level. For example, ischemic hypoxia induces the expression of HSPs, thus reducing reperfusion injury[22-24]. In myocardial hypertrophy, stress induces increased Hsp70 expression, thus providing a protective effect on the body; BBR may increase the expression of Hsp70 and inhibit cardiac hypertrophy by down-regulating miRNA-199a-5p.

Thus, the molecular mechanisms behind the effects of BBR treatment on cardiac hypertrophy are related to down-regulation of miRNA-199a-5p and increasing the expression of Hsp70. Since the expression of Hsp70 is regulated by a variety of miRNAs, whether BBR also regulates the expression of Hsp70 through other miRNAs remains for further study.

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