The Effects of Dracocephalum Heterophyllum Benth Flavonoid on Hypertrophic Cardiomyocytes Induced by Angiotensin II in Rats

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background:
Dracocephalum heterophyllum Benth flavonoid (DHBF) is a Tibetan and Uighur traditional medicine used to treat various disorders such as hypertension, lung heat, cough, and bronchitis; it has good antioxidant activity. Previous studies have shown that DHBF can reduce blood pressure in renovascular hypertensive rats, improve left ventricular systolic and diastolic function, and improve myocardial contractility. Therefore, we aimed to study the effect of DHBF on cardiomyocyte hypertrophy in cultured cells.

Material/Methods:
Neonatal rat cardiomyocytes were cultured, and hypertrophy was induced by angiotensin II (Ang II), with or without varying concentrations of the DHBF extract. Cell Counting Kit-8 assay was used to assess cell viability, RT-qPCR was used to determine mRNA levels, confocal laser scanning microscopy was used to measure cell surface area and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), and colorimetric assays were used to assess nitric oxide (NO) levels and nitric oxide synthase (NOS) activity.

Results:
Ang II treatment of cardiomyocytes reduced cell viability to ~75% that of controls. Ang II treatment also increased cell surface area; increased mRNA expression of c-jun, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC); increased $[Ca^{2+}]_i$; and reduced NOS activity and NO production. DHBF treatment could reverse these effects in a concentration-dependent manner.

Conclusions:
These results showed that DHBF can ameliorate cardiomyocyte hypertrophy induced by Ang II, as indicated by the downregulation of cardiac hypertrophy genes (*ANP*, *BNP*, and β-*MHC*) and reduction in cell surface area. The mechanism may be related to NO release and $[Ca^{2+}]_i$ regulation.

MeSH Keywords:
Angiotensin Amide • Hypertrophy • Myocytes, Cardiac

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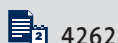
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Background

In developed countries, 1–2% of adults suffer from heart failure, and the proportion can be >10% in people older than the age of 70 [1]. Brouwers et al. reported that the 5-year survival rates for men and women with heart failure were 25% and 38%, respectively [2]. In recent years, comprehensive treatments, such as diuretics, angiotensin converting enzyme inhibitors, β-blockers, aldosterone antagonists, and cardiac resynchronization therapy, have improved the prognosis of heart failure patients and decreased their hospitalization rates, but morbidity and mortality are still high. Cardiac remodeling is the basic mechanism of heart failure, and cardiac hypertrophy is an important part of cardiac remodeling. Myocardial hypertrophy, a compensatory mechanism in response to pressure overload, occurs slowly, often accompanied by increased protein synthesis, structural remodeling, and cardiac organelle dysfunction. Cardiomyocyte hypertrophy involves increases in cell volume, in sarcomeric and embryonic gene expression, and in collagen fiber deposition. Hypertrophy can occur with congenital heart disease, hypertension, myocardial infarction, heart failure, and other cardiovascular diseases which share common pathological processes [3,4]. At the molecular level, the expression pattern of atrial natriuretic peptide (ANP) in myocardial cells changes, brain natriuretic peptide (BNP) production increases, the embryonic gene β-myosin heavy chain (β-MHC) activates, the expression of adult genes decreases, and myocardial protein synthesis increases. Cardiomyocytes are highly differentiated and cannot proliferate; they can only increase their volume. At the same time, the proliferation of fibroblasts, endothelial cells, and other non-myocytes can lead to other changes such as excess collagen deposition. At the organ level, there is an increase in cardiac mass, thickening of the ventricular wall, and enlargement of the heart cavity, which can manifest as diastolic dysfunction and reduced cardiac compliance [5]. Angiotensin II (Ang II) is an important initiation factor of myocardial hypertrophy [6]. Nitric oxide (NO), can cause vasodilation and reduce myocardial hypertrophy [7,8]. At the same time, myocardial hypertrophy is often accompanied by decreased sarcoplasmic reticulum calcium pump (Ca^{2+}-ATPase) activity and disorders of Ca^{2+} handling [9].

Dracocephalum heterophyllum is a genus of perennial Labiatae plants; it is also known as white flower branches, *Prunella vulgaris*, B. Paul (Tibetan name), and Xinjiang Uighur Mar Xan Gusi, Zupal. It is distributed in the areas of Shanxi, Inner Mongolia, Qinghai, Tibet, and Xinjiang, and is especially abundant in the village of Akto in the Autonomous Prefecture Tucker Kyrgyzstan of Xinjiang. Its whole grass contains volatile oils, flavonoids, and other constituents [10]. The aboveground part of the plant has good antibacterial and antioxidant activity, is used for myocardial protection, and treatment of coronary heart disease, and it lowers blood pressure. It is a traditional Uygur and Tibetan medicine for treating cough and stomach disease [11], but is also used to treat jaundice, fever, liver heat, headache, nebula, oral ulcers, and bronchitis [12]. *Dracocephalum heterophyllum* Benth flavonoid (DHBF) has been shown to relax blood vessel constriction induced by Ang II [13]. Both endothelium-intact and endothelium-denuded vessels have been shown to be affected, but the vasodilator action was stronger with the endothelium intact. NO and prostacyclin signaling pathways were involved in the relaxation effect of DHBF on the vascular endothelium, but the vasodilatory effects of DHBF likely involved additional mechanisms.

We assumed that this was related to L-type calcium channels and the concentration of NO [14]. Given the involvement of calcium channels and NO in myocardial hypertrophy, we postulated that DHBF could also affect cardiomyocyte hypertrophy, and could be developed into a new drug to treat cardiac hypertrophy. Therefore, the objective of this study was to evaluate the inhibitory effect of DHBF on cardiomyocyte hypertrophy induced by Ang II and to observe the effects of DHBF on intracellular calcium concentration ($[Ca^{2+}]_i$), activity of nitric oxide synthase (NOS), and production of NO. By performing this study, we will be able to investigate the preventive effects of DHBF on myocardial hypertrophy at the cellular level.

Material and Methods

Experimental animals

Sprague Dawley neonatal rats, up to 3 days old, were provided by the experimental animal center of the Xinjiang Medical University (License number: SCXK (New) 2003-0001). The rats had been housed in a specific pathogen free (SPF) environment in Xinjiang Medical University (License number: SCXK (New) 2011-0004).

Reagents and major equipment

Valsartan was purchased from the Sino-US Shanghai Shi Guibao Co., Ltd. (Shanghai, China). Double antibody, glutamine, D-Hanks solution, trypsin, 5-bromodeoxyuridine (BrdU), and Cell Counting Kit 8 (CCK-8) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Ang II was purchased from Aladdin Technologies Ltd. (Chengdu, China). Type II collagenase was purchased from Worthington Biochemical Corp. (Lakewood, NJ, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were products of Gibco Corporation (Thermo Fisher Scientific, USA). Both DyLight® 594-labeled donkey anti-mouse and anti-cardiac troponin T antibodies were purchased from Abcam (Cambridge, UK). Donkey serum was purchased from Beijing Iptonic Technology Ltd. (Beijing, China). Triton™ X-100 was purchased from Bio Engineering Co., Ltd. (Shanghai, China), and the reverse transcriptase kit was purchased from Thermo Science and Technology Co., Ltd. (Shanghai, China). The

NOS kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Both the Ca^{2+} fluorescent probe, Fluo-8 AM, and the NO assay kit were purchased from the Beyotime Biotechnology Research Institute (Jiangsu, China). In addition, the following reagents and equipment were used: quantitative PCR kit (Applied Biosystems, USA), LSM 710 laser confocal microscope (LEICA, Germany), reverse transcription (Bio-Rad), and quantitative PCR instrument (Applied Biosystems® 7500).

Plant material

DHBF was collected from Xinjiang Gkyzyl SuKeerg yrgyz Autonomous Prefectures, the Aketao county of Tucker village in 7 to 8 months in 2016. The plants were dried, crushed, and weighed by the Xinjiang Technical Institute of Physics and Chemistry in the Xinjiang Chinese Academy of Sciences. They were then immerged into 50 volumes of 70% alcohol for 2 hours, followed by 3 reflux extractions; the extracts were combined. AB-8 macroporous adsorption resin was used to purify the DHBF. The bound DHBF was removed by hot solvent, filtering the mixture to remove insoluble resin. A separating agent was added, the mixture was cooled, and brown crystals precipitated. The DHBF precipitate was collected by centrifugation, and was dried at 70°C. The yield was 3.76 mg·g^{-1}, and the total flavonoid content was 0.354 mg·L^{-1}.

Isolation and culture of primary cardiac myocytes from neonatal rats [15]

The rats were subjected to an aseptic thoracotomy; the hearts were quickly removed and placed into D-Hanks solution at 4°C. One-third to one-half of the atrial portion of the hearts was removed and discarded. The remaining apical ventricular tissue was minced and left at 4°C overnight in 0.1% trypsin solution. The next day, the digestion was continued with 0.08% type II collagenase, the cells were passed through a 200-mesh nylon screen and incubated in a 100 mm Petri dish. After 90 min, the media and nonadherent cells were collected and incubated with 0.1 mmol·L^{-1} BrdU to inhibit non-myocyte cell proliferation. The cardiomyocytes were cultured in 25 mm culture flasks at 3×10^6/mL, 60 mm Petri dishes at 1.5×10^6/mL, 96-well plates at 3×10^5/mL, and 6-well plates at 1×10^6/mL. The cells were cultured in DMEM containing 10% calf serum for 48 hours, and then in serum-free DMEM. The incubator was set at 5% CO_2 and 37°C. A single cell synchronized beat was observed using microscopy, and the frequency was ~70–90 contractions/min. The cardiomyocytes were used for the following experiments.

Experimental grouping

Experimental grouping included 1) control; 2) Ang II; 3) Ang II+valsartan (50 µmol·L^{-1}); 4) Ang II+DHBF (10 µmol·L^{-1}); 5) Ang II+DHBF (25 µmol·L^{-1}); 6) Ang II+DHBF (50 µmol·L^{-1}).

Model of myocardial hypertrophy and treatments

The cultured neonatal rat cardiomyocytes were treated with Ang II (1 µmol·L^{-1}) [16] for 96 hours to induce hypertrophy. The cells were also treated with different concentrations of DHBF (10, 25, or 50 µmol·L^{-1}) or valsartan (50 µmol·L^{-1}) for 30 min prior to the addition of Ang II. After 96 hours, the cells were analyzed for indexes of hypertrophy, as indicated below. Untreated cells served as normal controls.

Cell viability assay

Cardiomyocyte viability was measured by the CCK-8 method in 96-well plates. After treatments, 0.5% dimethyl sulfoxide (DMSO) was added to control cells; the other groups were washed 3 times with phosphate-buffered saline (PBS), 10 µL CCK-8 solution was added, and the cells were incubated at 37°C for 2 hours. The absorbance was then measured at a wavelength of 430 nm. Wells without cells were used for blank readings. There were 5 replicates per experiment; the experiment was repeated 3 times (n=3), and an average value was obtained. Cell survival rate (%)=($A_{test\ group}$ – A_{blank})/($A_{control\ group}$ – A_{blank})×100%.

RT-qPCR to detect expression of c-jun, ANP, BNP, and β-MHC mRNA as markers of hypertrophy

Myocytes were incubated and treated in 60 mm Petri dishes. After treatment, total RNA was isolated with TRIzol reagent, following manufacturer's instructions. Total RNA in diethyl pyrocarbonate (DEPC)-treated water was quantified by its A_{260} and A_{260}/A_{280} was used to estimate its purity. RNA was reversed transcribed into cDNA. One µg cDNA was subjected to qPCR with the following cycling parameters: 95°C for 30 sec, and then 40 cycles of 95°C for 5 sec, and 62°C for 20 sec. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalizing, and the relative expression level of the target genes were calculated by 2$^{-\Delta\Delta ct}$. The qPCR primers were designed by Shanghai Biotechnology Co., Ltd. and are shown in Table 1.

Confocal laser scanning microscopy and detection of myocardial cell surface area

Cardiomyocytes were cultured in 6-well plates and treated with reagents for 96 hours. The cells were removed from the plate, washed 3 times with PBS, and fixed for 15 min with 4% paraformaldehyde. The cells were then permeabilized for 10 min with 0.25% Triton X-100, blocked with donkey serum at room temperature for 30 min, and then incubated at room temperature for 1 hour with antibodies against cardiac troponin T. After washing, the cells were incubated at room temperature for 1 hour (protected from light) with DyLight 594-labeled secondary antibodies, and then were stained for 3 min

Table 1. Primer sequences for RT-qPCR.

	Target gene primer sequence (forward/reverse, F/R) (rat)	GenBank accession number	Length (bp)
c-jun	F: CGCGGGAGCCAACCAACGTG R: GCGTCCCCGCTTCAGTAACAAAGT	NM_021835.3	141
ANP	F: AGGAGAAGATGCCGGTAGAAGA R: GCTTCCTCAGTCTGCTCACTCA	NM_012612.2	145
BNP	F: TAGCCAGTCTCCAGAGCAATTC R: TTGGTCCTTCAAGAGCTGTCTC	NM_031545.1	153
β-MHC	F: GGAGATTACTGCCCTGGCTCCTA R: GACTCATCGTACTCCTGCTTGCTC	NM_017240.2	150
GAPDH	F: AAATGGTGAAGGTCGGTGTGAAC R: CAACAATCTCCACTTTGCCACTG	NM_017008.4	118

with 4',6-diamidino-2-phenylindole (DAPI, 1 g·mL^{-1}). Images were obtained by confocal laser microscopy (LSM 710). There were 3 replicates in each of 3, independent experiments. Five different visual fields were randomly selected from each sample, and 10 cells were quantified from each field. Image-Pro 6 software was used to measure the cell surface area and the average relative surface area was calculated.

Measuring [Ca^{2+}]$_i$

Myocytes were cultured and treated in 6-well plates. After washing 3 times with PBS, they were incubated with a calcium ion probe (Fluo-8 AM, final concentration of 5 µmol·L^{-1}) in a 5% CO$_2$ atmosphere at 37°C for 45–60 min. After washing 3 times with PBS, the cells were analyzed by dynamic scanning using laser scanning confocal microscopy. Twenty cells were randomly selected in each group and their intracellular calcium fluorescence intensity was quantified with software.

Determining the concentration of NO in the media and activity of NOS in cell supernatants

Myocytes were incubated in 25 mm culture flasks. After treatment, both the media and cell lysates were collected. NO and NOS were measured by colorimetric methods, strictly in accordance with the kit instructions. The absorbance at 540 nm was used to measure the presence of NO$_2^-$ after reaction with the Griess reagent; the level of NO was calculated with a standard curve. The activity of NOS was measured by the absorbance at 530 nm, following the kit instructions.

Statistical analyses

The data were analyzed using SPSS 17.0 statistical software. The data are expressed as means ± standard deviation (SD) (n=3). The *t*-test was used to compare 2 groups. One-way ANOVA was utilized for multi-group comparisons, with Student-Newman-Keuls testing for subsequent post-hoc comparisons

between 2 groups. Differences were considered statistically significant at *P*<0.05.

Results

Effect of DHBF on cell survival

Cell survival was detected by using the CCK-8 method. First, primary cardiomyocytes were treated with 10, 25, 50, 75, or 100 µmol·L^{-1} DHBF for 48 hours. Compared with that of the control group (no DHBF), 10–50 µmol·L^{-1} DHBF had no significant effect on the survival of the myocytes (*P*>0.05), but the cell survival was reduced significantly with concentrations of DHBF of 75 and 100 µmol·L^{-1} (*P*<0.05) (Figure 1A). Therefore, we selected concentrations of DHBF in the range of 0–50 µmol·L^{-1} in subsequent experiments. Second, cardiomyocytes were treated for 96 hours with different concentrations of DHBF (10, 25, or 50 µmol·L^{-1}) or valsartan (50 µmol·L^{-1}) and Ang II (1 µmol·L^{-1}). Compared with that of the corresponding control group (no DHBF), the cell survival was reduced significantly when the cells were treated with Ang II. The cell survival increased significantly with DHBF co-treatment, with 25 and 50 µmol·L^{-1} DHBF showing the effect (*P*<0.05) (Figure 1B). Compared with that of the valsartan and Ang II group, the cell survival increased with DHBF co-treatment (50 µmol·L^{-1}), but the difference was not significant (*P*>0.05). These results showed that the survival of cardiomyocytes, which was reduced by Ang II treatment, could be improved with DHBF co-treatment.

Effect of DHBF on mRNA expression of c-jun, ANP, BNP, and β-MHC in cardiomyocytes

RT-qPCR detection and analysis are shown in Figure 2. Primary myocytes were treated with Ang II (1 µmol·L^{-1}) for 48 or 96 hours. Compared with that of the control group, the expressions of c-jun, BNP, ANP, and β-MHC were upregulated 1.43±0.23, 1.26±0.31, 1.30±0.27, and 1.17±0.18, respectively, after Ang
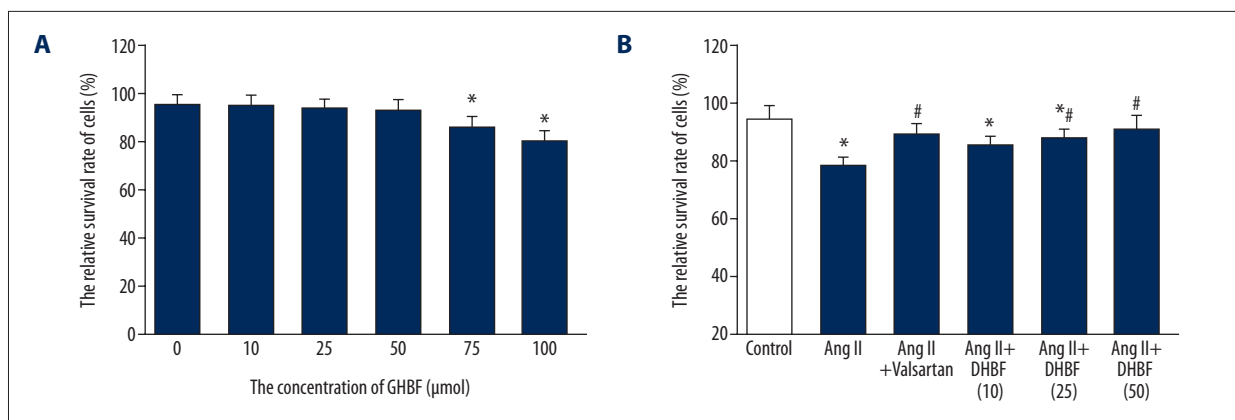
Figure 1. (**A**) Effects of different concentrations of DHBF on the survival of myocytes. N = 3. Data are expressed as means ±SD. * $P<0.05$ *vs.* 0 μmol·L^{-1} DHBF. DHBF, *Dracocephalum heterophyllum* Benth flavonoid. (**B**) Effects of different concentrations of DHBF on the viability of myocytes treated with Ang II. Concentration of DHBF is given in parentheses. Data are expressed as means ±SD. * $P<0.05$ *vs.* control; # $P<0.05$ *vs.* Ang II. Ang II – angiotensin II; DHBF – *Dracocephalum heterophyllum* Benth flavonoid; SD – standard deviation.
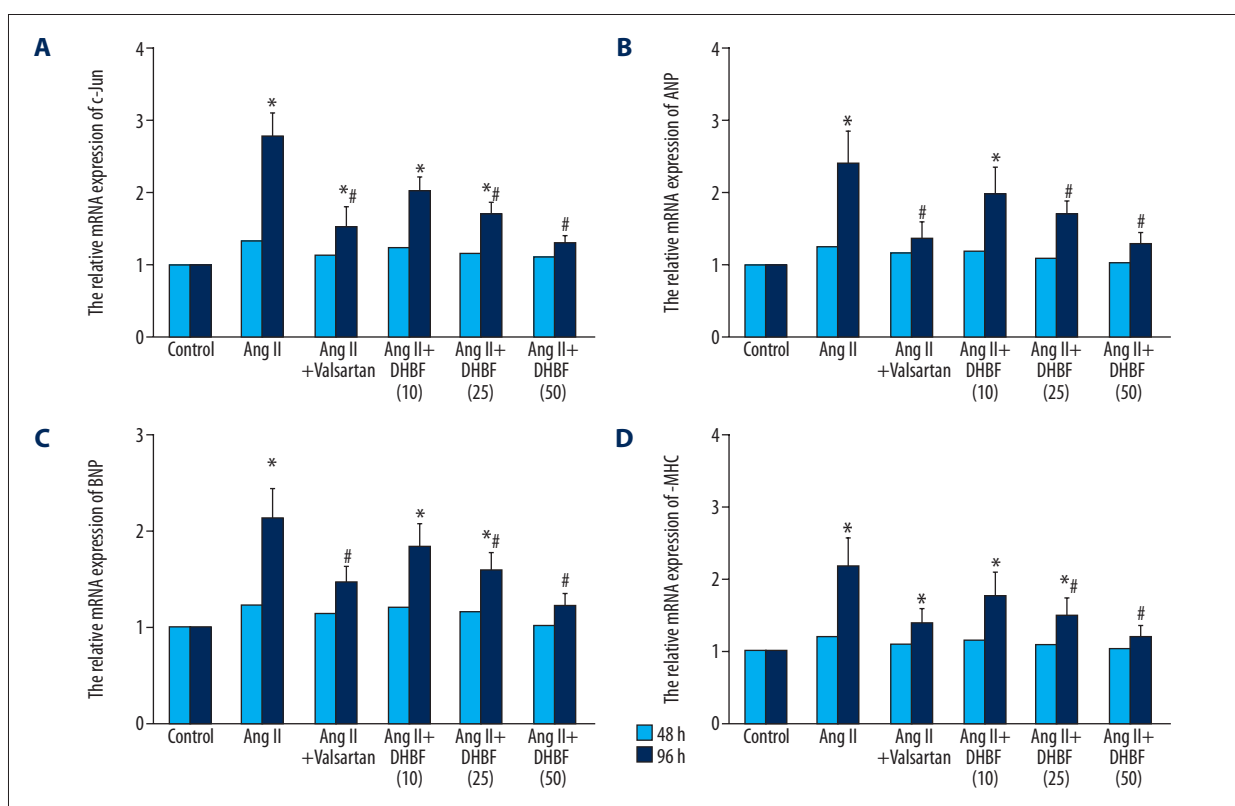

Figure 2. Effects of different concentrations of DHBF on mRNA expression of myocytes treated with Ang II. (**A**) c-jun; (**B**) ANP; (**C**) BNP; (**D**) β-MHC. Concentration of DHBF is given in parentheses. Data are expressed as means ±SD. * $P<0.05$ *vs.* control; # $P<0.05$ *vs.* Ang II. Ang II – angiotensin II; DHBF – *Dracocephalum heterophyllum* Benth flavonoid; SD – standard deviation.

II treatment for 48 hours, but there were no significant differences among the groups (means ±SD, n=6, $P>0.05$). After 96 hours of Ang II treatment, the upregulations were statistically significant at 1.78±0.31, 1.41±0.45, 1.11±0.32, and 1.18±0.38, respectively (means ±SD, n=6, $P<0.05$). However, the myocytes that were co-treated with DHBF showed a significant reduction in the expression of c-jun, ANP, BNP, and β-MHC mRNA, in a DHBF concentration-dependent manner ($P<0.05$).
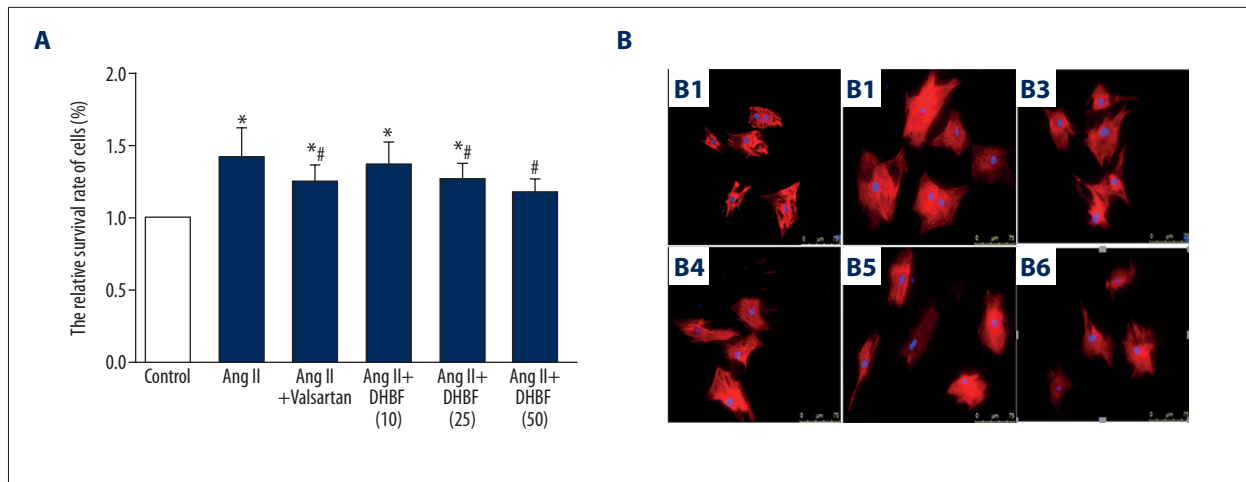
Figure 3. (**A**) Effects of different concentrations of DHBF on surface area of myocytes treated with Ang II. Concentration of DHBF is given in parentheses. Data are expressed as means ±SD. * $P<0.05$ *vs.* control; # $P<0.05$ *vs.* Ang II. Ang II – angiotensin II; DHBF – *Dracocephalum heterophyllum* Benth flavonoid; SD – standard deviation. (**B**) Fluorescence microscopy of myocytes: (**B1**) untreated cells; (**B2**) Ang II; (**B3**) Ang II+DHBF (10 µmol·L⁻¹); (**B4**) Ang II+DHBF (25 µmol·L⁻¹); (**B5**) Ang II+DHBF (50 µmol·L⁻¹). Magnification: 400×. Ang II – angiotensin II; DHBF – *Dracocephalum heterophyllum* Benth flavonoid.

Compared with that of the valsartan and Ang II group, the myocytes that were co-treated with DHBF (50 µmol·L⁻¹) showed a significant reduction in the expression of ANP and BNP ($P<0.05$), but no significant reduction in the expression of c-jun and β-MHC mRNA ($P>0.05$). The results showed that the increase in expressions of c-jun, BNP, ANP, and β-MHC mRNA in cardiomyocytes, which were induced by Ang II, could be inhibited by DHBF co-treatment, and that 50 µmol·L⁻¹ DHBF showed a stronger effect than did valsartan.

Effect of DHBF on the surface area of cardiomyocytes

The surface area of the myocytes in each group is shown in Figure 3A and 3B. Compared with that of the control group, the surface area of the cells was significantly increased in the Ang II groups ($P<0.05$). Compared with that of the Ang II-treated groups, the surface area of the myocytes was significantly reduced when they were co-treated for 96 hours with different concentrations of DHBF (10, 25, and 50 µmol·L⁻¹) ($P<0.05$). Compared with that of the valsartan and Ang II group, the surface area of myocytes was significantly reduced with 50 µmol·L⁻¹ DHBF ($P<0.05$). The results showed that the surface area of the myocytes, which was increased by Ang II, could be reduced by DHBF co-treatment, and the 50 µmol·L⁻¹ DHBF showed a stronger effect than did valsartan.

Effect of DHBF on the [Ca²⁺]ᵢ in cardiomyocytes stimulated with Ang II

The results are shown in Figure 4A and 4B. Compared with that of the control group, the [Ca²⁺]ᵢ of cardiomyocytes was significantly increased when they were stimulated with Ang

II (1 µmol·L⁻¹) ($P<0.05$). Compared with that of the Ang II-stimulated groups, [Ca²⁺]ᵢ was significantly reduced when the cells were co-treated for 96 hours with different concentrations of DHBF (10, 25, and 50·µmol·L⁻¹) ($P<0.05$). Compared with that of the valsartan and Ang II group, [Ca²⁺]ᵢ was significantly reduced with DHBF (50 µmol·L⁻¹) ($P<0.05$). The results showed that the increase in [Ca²⁺]ᵢ induced by Ang II treatment could be inhibited by DHBF, and that 50 µmol·L⁻¹ DHBF showed a stronger effect than did valsartan.

Effect of DHBF on NO production and the activity of NOS in cardiomyocytes treated with Ang II

Compared with that of the control group, the concentration of NO in the media and the activity of NOS in the myocytes were significantly decreased when the cells were treated with Ang II ($P<0.05$). Compared with that of the Ang II-treated cells, the concentration of NO and the activity of NOS were increased when the cells were co-treated for 48 hours with different concentrations of DHBF (10, 25, and 50 µmol·L⁻¹), but there was no significant difference among the groups ($P>0.05$). However, the concentration of NO and the activity of NOS were significantly increased when the cells were co-treated for 96 hours with different concentrations of DHBF (10, 25, and 50 µmol·L⁻¹) ($P<0.05$). Compared with that of the valsartan and Ang II group, the concentration of NO and the activity of NOS were significantly increased with DHBF (50 µmol·L⁻¹) ($P<0.05$). These results showed that the concentration of NO and the activity of NOS associated with hypertrophied cardiomyocytes can be increased by DHBF co-treatment, and that 50 µmol·L⁻¹ DHBF showed a stronger effect than did valsartan (Figure 5A, 5B).
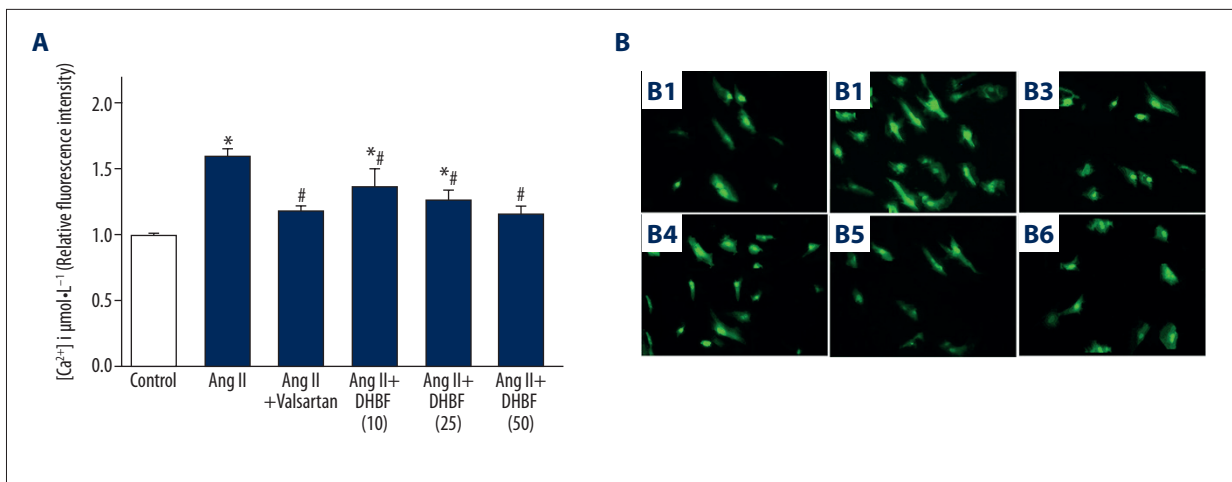
Figure 4. (**A**) Effects of different concentrations of DHBF on $[Ca^{2+}]_i$ of myocytes induced by Ang II. Concentration of DHBF is given in parentheses. Data are expressed as means ±SD. * $P<0.05$ *vs* control; # $P<0.05$ *vs.* Ang II. Ang II – angiotensin II; DHBF – *Dracocephalum heterophyllum* Benth flavonoid; SD – standard deviation. (**B**) Fluorescence microscopy on $[Ca^{2+}]_i$ of myocytes: (**B1**) untreated cells; (**B2**) Ang II; (B3) Ang II+valsartan (50 µmol·L⁻¹); (**B4**) Ang II+DHBF (10 µmol·L⁻¹); (**B5**) Ang II+DHBF (25 µmol·L⁻¹); (**B6**) Ang II+DHBF (50 µmol·L⁻¹). Magnification: 200×. Ang II – angiotensin II; DHBF – *Dracocephalum heterophyllum* Benth flavonoid.
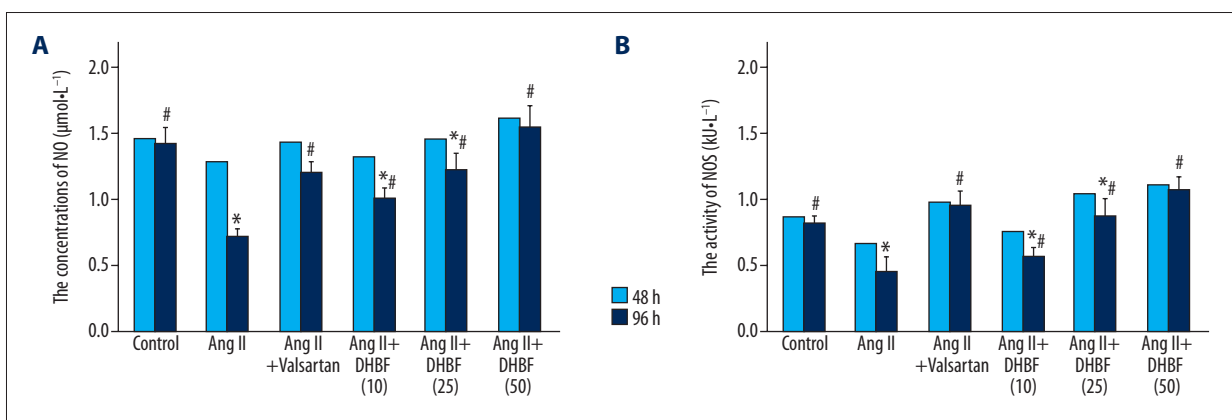

Figure 5. (**A**) Effects of different concentrations of DHBF on NO production in hypertrophic cardiomyocytes induced by Ang II. Ang II – angiotensin II; DHBF – *Dracocephalum heterophyllum* Benth flavonoid; NO – nitric oxide. (**B**) Effects of different concentrations of DHBF on the activity of NOS in hypertrophic cardiomyocytes induced by Ang II. Ang II – angiotensin II; DHBF – *Dracocephalum heterophyllum* Benth flavonoid; NOS – nitric oxide synthase.

Discussion

The consensus in the medical profession has long been that cardiac hypertrophy, which manifests as an increase in cardiomyocyte protein and volume, is a major risk factor for cardiovascular disease in humans. Hypertrophy is often associated with changes in stromal components, decreased cardiac compliance, and decreased circulatory function; many factors are involved in the formation of myocardial hypertrophy [18]. There are many treatment methods, but the outcome is still not ideal. In view of this, it is very important to find more effective drugs and targets to inhibit cardiac hypertrophy. Myocardial hypertrophy is a complex and dynamic process that involves many

factors. It has been shown that phosphatidylinositol 3-kinase/protein kinase B, calcineurin, mitogen-activated protein kinase (MAPK), NO/cGMP, and many other signaling pathways are involved in this pathological process [18]. The renin-angiotensin-aldosterone system (RAAS) and renin-catecholamine system can directly cause the development of cardiac hypertrophy.

In our study, we showed that there was a trend toward decreased expression of c-jun, ANP, BNP, and β -MHC mRNA; increased concentration of NO; and activity of NOS in cardiomyocytes after 48-hour induction by Ang II and DHBF. This may be due to the fact that the stimulation time of Ang II was insufficient to induce hypertrophy, but it may also be related to

the protective effect of DHBF on cardiomyocytes. However, after 96 hours of co-treatment with DHBF and Ang II, the expression of c-jun, ANP, BNP, and β-MHC decreased significantly, and the concentration of NO and the activity of NOS increased significantly, suggesting that DHBF could inhibit cardiomyocyte hypertrophy induced by Ang II.

Ang II is the main effector molecule of RAAS. It binds to the AT1 receptor (a Gq family receptor) that is on the myocyte cell membrane, activating intracellular signaling pathways [19]. In this study, primary myocardial hypertrophy was induced by Ang II, which is commonly used as a model to evaluate cardiac hypertrophy *in vitro* and can exclude factors such as hemodynamic stress and other neurohumoral stimulation. By observing the mRNA expression of the proto-oncogene c-jun, cardiac hypertrophy markers, ANP, BNP, and β-MHC, as well as the surface area of the myocytes, it was confirmed that the model of cardiomyocyte hypertrophy was successful. At the same time, we observed that DHBF could significantly increase the survival of the myocytes treated with Ang II, inhibit the expression of the mRNA for c-jun, ANP, BNP, and β-MHC, and reduce the surface area of the cells, suggesting that DHBF plays an obvious preventive role in cardiomyocyte hypertrophy.

We used Ang II as an effector molecule to induce cardiomyocyte hypertrophy. Many experiments have shown that Ang II has strong inotropic effects and plays a role in vascular contraction. It can lead to increased cell protein synthesis and participates in the early process of signal transduction leading to myocyte hypertrophy. Ang II can bind to its receptor, AT1, activating phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to form inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 can increase $[Ca^{2+}]_i$ and DAG can activate protein kinase C (PKC). Increased Ca^{2+} and activation of PKC can lead to the early upregulation of proto-oncogenes, such as c-fos, c-myc, c-jun, and Egr-1; these genes encode proteins that act as third messengers that regulate the expression of target genes, which results in cardiac hypertrophy [20]. At the same time, Ang II could upregulate L-type-calcium channels, increasing calcium influx [21]. The results showed that the protective effect of DHBF on myocardial hypertrophy may be related to blocking the AT1 receptor and L-type calcium channels.

In our study, Ang II induced cardiomyocyte hypertrophy obviously, which was characterized by increased content of intracellular protein and increased cell volume, but the number of cells did not change, indicating no effect on cell proliferation. This can lead to increased myocardial contractility. Both the NO concentration and NOS activity decreased in the model group of myocardial hypertrophy, showing that Ang II not only can induce myocardial hypertrophy, but also can reduce the activity of NOS. These effects may be related to α1 adrenergic receptors

and are mediated by the G protein that is insensitive to pertussis toxin. Adding exogenous NO was shown to prevent cardiac hypertrophy induced by Ang II, suggesting that NO not only decreases coronary vasodilation and increases coronary blood flow and myocardial contractility, but also plays an important role in preventing myocardial hypertrophy [22]. In our previous study, we found that DHBF can reduce blood pressure in renovascular hypertensive rats and can improve left ventricular systolic and diastolic function and myocardial contractility; the mechanism of DHBF may be related to decreased endothelin levels in the blood, cardiac tissue, and renal tissue, as well as increased levels of NO [8]. Our current experimental results showed that the level of NO in the DHBF group was higher than that in the model group. DHBF could inhibit cardiomyocyte hypertrophy induced by Ang II, and at the same time increase the activity of NOS in the cardiomyocytes. The resulting increased concentration of NO in the heart could elevate diastolic coronary blood flow, which could inhibit cardiac hypertrophy. These results showed that the protective effect of DHBF on cardiac hypertrophy may be related to the concentration of NO in the heart.

Increased $[Ca^{2+}]_i$ is a central link in the development and progression of myocardial hypertrophy induced by external stimuli and/or intrinsic dysfunction [9]. During myocardial excitation and contraction, the calcium current is the main current of the action potential plateau, and Ca^{2+} directly combines with muscle filaments to induce myocardial contraction. When the concentration of intracellular Ca^{2+} drops, Ca^{2+} dissociates from troponin and induces myocardial relaxation. Ca^{2+} is the most important intracellular second messenger, transmitting a variety of biological signals through the cytoplasm to regulate cell growth and development, including the regulation of cardiac function [23]. Ca^{2+} is also an important signaling component for cardiomyocyte hypertrophy; calmodulin (CaM) and CaM-dependent protein kinase (CaMKII) are important regulatory signals. Alterations in intracellular Ca^{2+} signaling are the primary stimulus for hypertrophic responses, which play a central role in cardiac hypertrophy and related gene expression. The calcineurin/nuclear factor of activated T cells (CaN/NFAT) pathway and the CaMKII/histone deacetylase (HDAC) pathway, which are Ca^2-dependent, are involved [24]. At the same time, decreased activity of Ca^{2+}-ATPase can lead to disorders of calcium handling, and calcium overload is an important pathophysiological mechanism leading to dysfunction of myocardial systole and diastole. Studies have shown that NO-cGMP-PKG inhibits the influx of Ca^{2+} in L-type calcium channels, inhibits the activation of CaN, and weakens NFAT nuclear translocation and transcriptional activity; these all can lead to inhibition of myocardial hypertrophy. CaMKIIs may play a role in cardiac hypertrophy by activating the CREBP transcription factor family [25]. In our study, the $[Ca^2]_i$ was significantly lower in the group co-treated with DHBF than in the model group. This showed that DHBF not only could inhibit cardiomyocyte

hypertrophy induced by Ang II, but it also could significantly decrease the $[Ca^{2+}]_i$ of myocardial cells. These results suggest that the regulation of $[Ca^{2+}]_i$ is one of the mechanisms of DHBF to inhibit myocardial hypertrophy.

In this study, the cells were primary cultured neonatal rat cardiomyocytes. Adult rat cardiomyocytes would more closely mimic the internal environment of the disease. If we could study cardiomyocytes from adult rats, the experimental design would be more rigorous. Valsartan is a commonly used drug for the treatment of cardiac hypertrophy clinically. In this study, by comparing the effects of DHBF and valsartan, we found that a high concentration of DHBF was superior to valsartan in inhibiting cardiac hypertrophy by decreasing myocyte surface area and reducing the expression of mRNA of the c-jun proto-oncogene and the cardiac hypertrophy markers, ANP, BNP, and β-MHC. This suggests that DHBF has potential for drug development. The mechanism by which DHBF inhibits myocardial hypertrophy and whether DHBF has an inhibitory effect in animal models of myocardial hypertrophy need to be studied further.

Conclusions

In summary, the data we obtained clearly showed that DHBF can increase the viability of hypertrophic cardiomyocytes, downregulate the mRNA expression of c-jun, ANP, BNP, and β-MHC, and decrease the surface area of hypertrophic cardiomyocytes, which were all induced by Ang II. These results suggest that DHBF plays a protective role in hypertrophic cardiomyocytes. Its mechanism of action might be related to promoting the release of NO, regulating $[Ca^{2+}]_i$, or other mechanisms and cellular signaling pathways that remain unknown. DHBF has the potential to be used as a drug for the prevention and treatment of heart failure.

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