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Original Article

Evodiamine Inhibits Angiotensin Ⅱ-Induced Rat Cardiomyocyte Hypertrophy*

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ABSTRACT **Objective:** To investigate the effects of evodiamine (Evo), a component of *Evodiaminedia rutaecarpa* (*Juss*.) Benth, on cardiomyocyte hypertrophy induced by angiotensin Ⅱ (Ang Ⅱ) and further explore the potential mechanisms. **Methods:** Cardiomyocytes from neonatal Sprague Dawley rats were isolated and characterized, and then the cadiomyocyte cultures were randomly divided into control, model (Ang Ⅱ 0.1 μmol/L), and Evo (0.03, 0.3, 3 μmol/L) groups. The cardiomyocyte surface area, protein level, intracellular free calcium ($[Ca^{2+}]_i$) concentration, activity of nitric oxide synthase (NOS) and content of nitric oxide (NO) were measured, respectively. The mRNA expressions of atrial natriuretic factor (ANF), calcineurin (CaN), extracellular signal-regulated kinase-2 (ERK-2), and endothelial nitric oxide synthase (eNOS) of cardiomyocytes were analyzed by real-time reverse transcription-polymerase chain reaction. The protein expressions of calcineurin catalytic subunit (CnA) and mitogen-activated protein kinase phosphatase-1 (MKP-1) were detected by Western blot analysis. **Results:** Compared with the control group, Ang Ⅱ induced cardiomyocytes hypertrophy, as evidenced by increased cardiomyocyte surface area, protein content, and ANF mRNA expression; increased intracellular free calcium ($[Ca^{2+}]_i$) concentration and expressions of CaN mRNA, CnA protein, and ERK-2 mRNA, but decreased MKP-1 protein expression ($P<0.05$ or $P<0.01$). Compared with Ang Ⅱ, Evo (0.3, 3 μmol/L) significantly attenuated Ang Ⅱ-induced cardiomyocyte hypertrophy, decreased the $[Ca^{2+}]_i$ concentration and expressions of CaN mRNA, CnA protein, and ERK-2 mRNA, but increased MKP-1 protein expression ($P<0.05$ or $P<0.01$). Most interestingly, Evo increased the NOS activity and NO production, and upregulated the eNOS mRNA expression ($P<0.05$). **Conclusion:** Evo significantly attenuated Ang Ⅱ-induced cardiomyocyte hypertrophy, and this effect was partly due to promotion of NO production, reduction of $[Ca^{2+}]_i$ concentration, and inhibition of CaN and ERK-2 signal transduction pathways.

KEYWORDS evodiamine, cardiomyocyte, hypertrophy, angiotensin Ⅱ, calcineurin, extracellular signal-regulated kinase-2, nitric oxide

Hypertension, atherosclerosis and many other cardiovascular diseases may finally lead to the development of cardiac hypertrophy. Cardiac hypertrophy is one of the most important compensatory responses of myocardium. Increased cardiac pressure, increased blood volume, and the neurohormonal dysregulation are important factors in developing cardiac hypertrophy.[1] Although cardiac hypertrophy is initially a process of adaptation, sustained hypertrophy can eventually become ventricular dilatation, heart failure and sudden death.[2] One of the most important factors contributing to the development of cardiomyocyte hypertrophy is angiotensin Ⅱ (Ang Ⅱ).[3] Ang Ⅱ increases the protein synthesis and cell volume in cultured neonatal rat cardiomyocytes through type 1 Ang Ⅱ receptor.[4]

It is reported that numerous molecular pathways are involved in hypertrophic signaling, including protein kinase C (PKC), mitogen-activated protein kinase (MAPK), c-Jun-N-terminal kinase/stress activated protein kinase (JNK/SAPK), and calcineurin (CaN) signal transduction pathways.[5,6] Calcium acts as the second messenger to initiate cardiac hypertrophy.[7,8] Sustained elevation in intracellular calcium activates CaN, followed by dephosphorylation of nuclear factor

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of activated T cells (NFAT3) in the cytoplasm. NFAT3 enters the nucleus and binds zinc finger transcription factors 4 (GATA4), induces the expression of genes responsible for cell proliferation, resulting in cardiac hypertrophy. MAPK is another important signal pathway leading to Ang Ⅱ-induced cardiac hypertrophy.[9] Phosphorylation of extracellular signal-regulated kinase (ERK) promotes cardiomyocyte hypertrophy.[10] It is known that nitric oxide (NO), an important transcellular signaling molecule, is a negative regulator of cardiomyocyte hypertrophy via cyclic guanosine monophosphate (cGMP) and cGMP-dependent protein kinase type Ⅰ (PKG Ⅰ) which can suppress NFAT transcriptional activity.[11] These signaling pathways play an integrated role in producing cardiomyocytes hypertrophy.

The dried, unripe fruit of *Evodia rutaecarpa*, has been used for thousands of years to treat gastrointestinal disorders, postpartum hemorrhage, headache, and cardiovascular diseases.[12,13] Evodiamine (Evo) is one of the major bioactive compounds isolated and purified from the unripe fruit of *Evodiaminedia rutaecarpa* (*Juss*.) Benth. Evo is an intriguing molecule and has been shown to have a variety of pharmacologic effects. These beneficial effects include anti-atherosclerosis, antiobesity, antitumor effects, and inhibition of corticosterone secretion.[14-17] We have demonstrated that the extracts of *Evodia rutaecarpa* could inhibit the monocrotaline-induced right ventricular hypertrophy in rats.[18] In the present study, we further investigated the effects of Evo on Ang Ⅱ-induced cardiomyocyte hypertrophy *in vitro*, focusing on signal transduction pathways as potential mechanisms.

METHODS

Animals and Reagents

Sprague-Dawley (SD) rats aged 7 weeks old (205–250 g, SPF Ⅱ grade, certificate No. SCXK 10-2006) were obtained from the Animal Center of Sichuan University (China). Adult and new-born SD rats were kept in a regulated environment (50%±2% humidity, 24±1 ℃) with 12 h light/dark cycle (light on 8:00 am–8:00 pm). All animal studies were in compliance with the "Guide for the care and use of laboratory animals" in China (No.14924, 2001) and approved by the Animal Ethics Committee of Zunyi Medical College (No. 266).

Evo (purity 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). Ang Ⅱ, antibodies of calcineurin A (CnA), mitogen-activated protein kinase phosphatase-1 (MKP-1) and 5'-bromodeoxyuridine (5'-BrdU) were purchased from Sigma-Aldrich (USA). Trypsin and Dulbecco modified Eagle medium (DMEM) were purchased from Gibco BRL (USA). Fetal bovine serum (FBS) was from Hanzhou Season's Co. (Hanzhou, China). Fluo-3/AM calcium indicator was purchased from Biotium (USA). Kits for the measurement of protein content and NO content were purchased from Beyotime Institute Biotechnology (China). Kit for the measurement of NO synthase (NOS) activity was purchased from Nanjing Jian Cheng Bio-engineering Institute (China). RNeasy mini kits and the primers of atrial natriuretic factor (ANF), CaN, ERK-2 and endothelial nitric oxide synthase (eNOS) were purchased from Takara Biological Technology (China). SYBR green PCR Master Mix was purchased from Applied Biosystems (USA).

Neonatal SD Rat Cardiomyocytes Culture, Identification, Grouping and Treatment

Primary cardiomyocytes from 1–3 days old neonatal SD rats were prepared, randomly divided into control, model (Ang Ⅱ 0.1 μmol/L), and Evo (0.03, 0.3, 3 μmol/L) groups. Then, cardiomyocytes were identified by α-smooth muscle actin (α-SMA) stain, and cultured for 48 h in DMEM containing 20% FBS and 0.1 mmol/L 5'-BrdU as described previously.[19] The cells were adjusted to 1×10^5–5×10^5 cells/mL for measuring cell surface area and intracellular free calcium concentration ($[Ca^{2+}]_i$). Cells were also adjusted to 1×10^6–3×10^6 cells/mL for measuring cellular total protein content, NO content, NOS activity, or eNOS mRNA and protein expressions. After 24 h of serum-free DMEM starvation, cardiomyocytes were pretreated with Evo for 30 min and subsequently were stimulated with 0.1 μmol/L Ang Ⅱ for additional 48 h.

Measurement of Cardiomyocyte Surface Area

To determine changes in cell size, the cardiomyocytes were fixed in 4% polyformaldehyde solution for 15 min and stained with hematoxylin and eosin (HE). The surface area of single cell was measured by the Leica Qwin V3 Imaging Analytic System. Five random fields (10–15 cells per field) from each slide were analyzed. The experiments were repeated 5 times.

Measurement of Cardiomyocyte Protein Level

Cells were washed 3 times with phosphate-buffered saline (PBS), then were homogenized with radio-immunoprecipitation assay (RIPA) lysis buffer and centrifuged at 11,000 r/min for 30 min at 4 ℃. The protein concentration in the supernatant was determined by the bicinchoninic acid (BCA) assay, and then the protein content per cell was calculated.

Measurement of $[Ca^{2+}]_i$ Concentration

After the treatment described above, the cardiomyocytes cultured in Petri dishes were washed 3 times with sterile PBS, then incubated with 5 μmol/L Fluo-3/AM for 50 min at 37 ℃. Cardiomyocytes were rinsed 3 times with PBS to remove the remaining dye, and further incubated in DMEM. Changes in the $[Ca^{2+}]_i$ concentration were represented as fluorescence intensity. The fluorescence intensity was measured by laser scanning confocal microscopy (Leica TCS SP8, Germany) with excitation at 488 nm and emission at 505–550 nm.

Measurement of NOS Activity and NO Content

NOS activity was measured using a NOS detection kit, according to the manufacturer's instruction. Formation of NO from L-arginine and oxygen is catalyzed by NOS. The NO and nucleophilic material produce a colored compound, the optical density values of the samples were determined on a spectrophotometer at 530 nm. Then NOS activity in the samples was calculated according to formulate of the instruction.

NO content derivative nitrite was determined in the supernatant with the Griess reagent. A nitrite detection kit was used according to the instruction provided by the manufacturer. Briefly, at room temperature, 50 μL of medium or standard $NaNO_2$ was mixed with 50 μL of Griess reagent Ⅰ and Griess Reagent Ⅱ in a 96-well plate. Then optical density was read at 540 nm in a microplate reader. The NO content in the samples was determined from a sodium nitrite standard curve.

Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis

Expressions of ANF, ERK-2, eNOS and CaN mRNA were determined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from primary cultured cardiomyocyte with Trizol reagent. RNA was quantified by measuring the optical density of samples at 260/280 nm with an ultraviolet spectrometry, dissolved in diethylpyrocarbonate-treated water, and stored at –80 ℃. Total RNA was then reverse-transcribed with MuLV reverse transcriptase and Oligo-dT primers. Sequences of primers used for real-time RT-PCR analyses were summarized in Table 1. Genes of interest were amplified with SYBR green PCR Mix. The cycle time (Ct) values of the target gene were normalized with that of β-actin. The data were expressed as percentage of controls.

Table 1. Sequences of Primers Used for Real-Time RT-PCR Analyses

Gene	GenBank access No.	Primer (5'–3')	Product size (bp)
β-actin	NM 031144	F-GGAGATTACTGCCCTGGCTCCTA	150
		R-GACTCATCGTACTCCTGCTTGCTG	
ANF	NM 012612	F-TGACAGGATTGGAGCCCAGAG	75
		R-TCGAGCAGATTTGGCTGTTATCTTC	
CaN	NM 017041	F-CTGAGATGCTGGTAAACGTCCTGA	122
		R-TGCTCGGATCTTGTTCCTGATG	
ERK-2	NM 053842	F-GGCACCAACCATTGAGCAGA	101
		R-GATCATTGCTGAGGTGCTGTGTC	
eNOS	NM 021838	F-AGCTGGATGAAGCCGGTGAC	164
		R-CCTCGTGGTAGCGTTGCTGA	

Western Blot Analysis

Protein (50 μg) was heat denatured at 100 ℃ for 5 min, electrophoretically separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk in tris-buffered saline Tween-20 (TBST) buffer for 2 h at room temperature and incubated with a primary mouse anti-rat CnA (1:600), anti-MKP-1 (1:500) or GAPDH (1:10000) antibody in 5% nonfat dry milk in TBST buffer at 4 ℃ overnight. Membranes were then washed 3 times for 10 min each with TBST buffer, and incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin G (1:10000) at room temperature for 2 h with gentle rotation. After further washing, bound secondary antibody was incubated with pierce chemiluminescence reagent. The image was scanned and band densities were quantified using Quantity One 1 Data Analysis Software v4.52.

Statistical Analysis

All the data were expressed as mean ±

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standard deviation ($\bar{x} \pm s$). The data was analyzed by one-way analysis of variance followed by multi-range tests, using the SPSS 13.0 for Windows statistical program. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Cardiomyocyte Identification

Isolated and cultured cardiomyocytes were identified by immunohistochemical stain with α-SMA. The nuclei were stained as light blue, and cytoplasm stained as brownish fibers. Under microscope, cardiomyocytes showed pulse beating. The prepared cardiomyocytes had a purity of 95% as we demonstrated previously.[19]

Evo Inhibits Cardiomyocyte Hypertrophy Induced by Ang Ⅱ

HE staining showed that the cardiomyocytes treated with Ang Ⅱ became swollen with enlarged unclear borders in the cells as compared to normal cardiomyocytes. Evo (0.03–3 μmol/L) markedly alleviated the morphological changes induced by Ang Ⅱ in a concentration-dependent manner (Figure 1).

Ang Ⅱ significantly increased the cardiomyocyte surface area by 176.8 % and protein content 18.0% ($P<0.01$). Evo (0.3 and 3 μmol/L) significantly attenuated these changes induced by Ang Ⅱ ($P<0.05$ or $P<0.01$). There was a low basal ANF mRNA expression in the cardiomyocytes. Ang Ⅱ increased the biomarker (ANF mRNA) for cardiomyocyte hypertrophy by 2.3-fold ($P<0.01$), which was significantly reduced by Evo treatment ($P<0.01$, Figure 2).

Evo Reduces Concentration of $[Ca^{2+}]_i$ in Ang Ⅱ-Induced Hypertrophy Cardiomyocyte

Ang Ⅱ (0.1 μmol/L) significantly increased $[Ca^{2+}]_i$ concentration of cardiomyocyte by approximately 60% ($P<0.01$). However, Evo (0.03–3 μmol/L) apparently blocked the $[Ca^{2+}]_i$ increase induced by Ang Ⅱ and the inhibition ratio were 12.7%, 24.3% and 34.7% with increasing Evo concentration, respectively ($P<0.05$ or $P<0.01$, Figure 3).

Effects of Evo on NO Content and NOS Activity after Ang Ⅱ Stimulation

Ang Ⅱ (0.1 μmol/L) significantly decreased the NO content and NOS activity ($P<0.05$ or $P<0.01$). Treatment with Evo (0.03–3 μmol/L) prevented Ang Ⅱ-induced decrease in NO content and NOS activity, respectively ($P<0.05$, Figure 4).

Effects of Evo on Ang Ⅱ-Induced mRNA Expressions of CaN, ERK-2 and eNOS

Ang Ⅱ significantly upregulated the mRNA expressions of CaN and ERK-2 by 3.2, 1.8-fold respectively, and downregulated eNOS mRNA expression by approximately 25% ($P<0.05$ or $P<0.01$). However, Evo (0.03–3 μmol/L) significantly suppressed the overexpression of CaN and ERK-2 mRNA, and increased the expression of eNOS mRNA ($P<0.05$ or $P<0.01$, Figure 5).

Effects of Evo on Ang Ⅱ-Induced Protein Expressions of CnA and MKP-1

Ang Ⅱ significantly increased the CnA protein expression by 2.2-fold and decreased the MKP-1 by 43.6% ($P<0.05$ or $P<0.01$). However, Evo (0.3 and

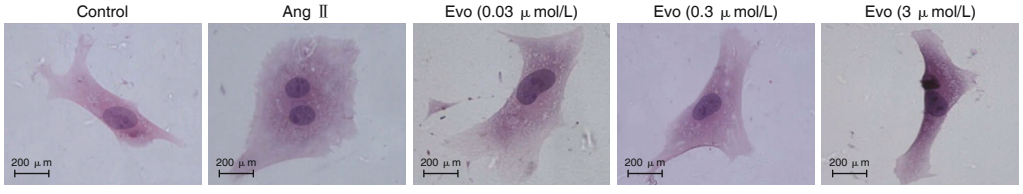

Figure 1. Effects of Evo on Ang Ⅱ- Induced Hypertrophy Cardiomyocytes (HE staining, ×400)

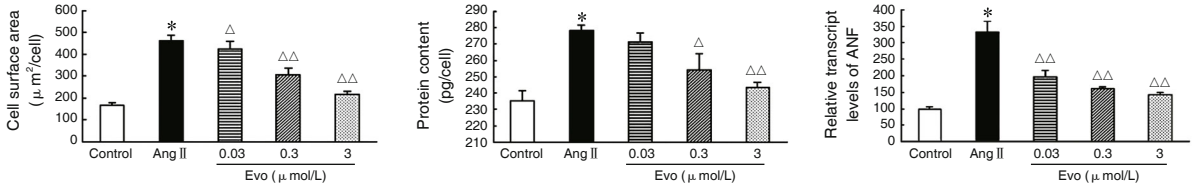

Figure 2. Effects of Evo on Cell Surface Area, Protein Content and ANF mRNA Expression of Hypertrophy Cardiomyocytes Induced by Ang Ⅱ (n=5, $\bar{x} \pm s$)

Notes: *$P<0.01$ *vs*. control group; △$P<0.05$, △△$P<0.01$ *vs*. Ang Ⅱ group

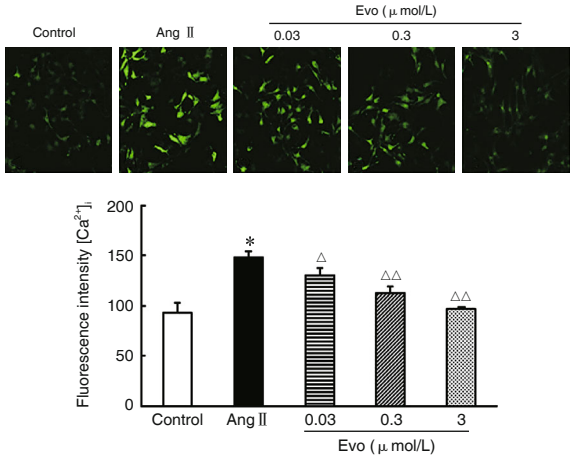

Figure 3. Effects of Evo on the $[Ca^{2+}]_i$ Fluorescence Intensity of Hypertrophy Cardiomyocytes Induced by Ang Ⅱ (n=5, $\bar{x} \pm s$)

Notes: $^{*}P$<0.01 *vs*. control group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. Ang Ⅱ group

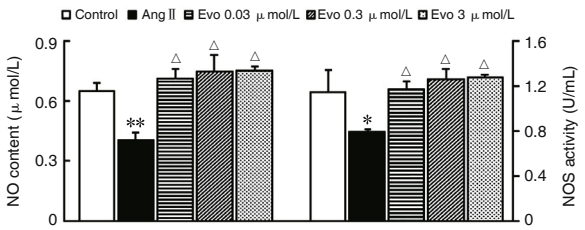

Figure 4. Effects of Evo on NO Content and NOS Activity (n=5, $\bar{x} \pm s$)

Notes: $^{*}P$<0.05, $^{**}P$<0.01 *vs*. control group; $^{\triangle}P$<0.05 *vs*. Ang Ⅱ group

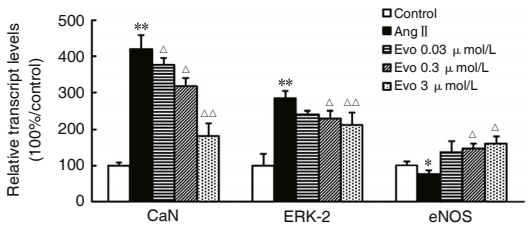

Figure 5. Effects of Evo on mRNA Expressions of CaN, ERK-2 and eNOS in Ang Ⅱ-Induced Hypertrophic Cardiomyocytes (n=4, $\bar{x} \pm s$)

Notes: $^{*}P$<0.05, $^{**}P$<0.01 *vs*. control group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. Ang Ⅱ group

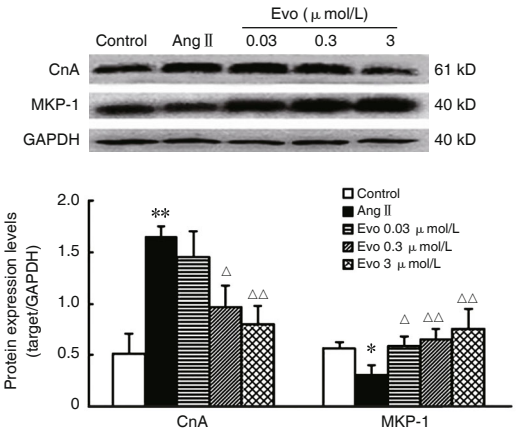

Figure 6. Effects of Evo on Protein Expressions of CnA and MKP-1 in Ang Ⅱ-Induced Hypertrophic Cardiomyocytes (n=3, $\bar{x} \pm s$)

Notes: $^{*}P$<0.05, $^{**}P$<0.01 *vs*. control group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. Ang Ⅱ group

3 μmol/L) suppressed the protein overexpression of CnA by 41.2% and 51.5%, and increased the MKP-1 protein expression by 109.7 % and 141.9 %, respectively (P<0.05 or P<0.01, Figure 6).

DISCUSSION

It has been shown that the extracts of *Evodia rutaecarpa* inhibit the monocrotaline-induced right ventricular hypertrophy in rats, probably mediated through inhibition of the CaN signaling pathway.[18] The current study further demonstrated that Evo is an active ingredient of *Evodia rutaecarpa* in preventing cardiomyocyte hypertrophy induced by Ang Ⅱ, as evidenced by morphology and the expression of cardiomyocyte hypertrophy biomarker ANF. We have also found that Evo prevents Ang Ⅱ-induced elevations of calcium concentrations in the cadiomyocytes, and Ang Ⅱ-induced increases in CaN mRNA and CaN protein expressions. In addition, Evo also affects on MAPK/ERK signal pathway, increases NO production by increasing NOS activity. All these effects would contribute to anti-cardiomyocyte hypertrophy effects of evodianmine.

It has been reported that the morphological changes of cardiomyocyte hypertrophy can be induced *in vitro* by stimulating cultured neonatal rat cardiomyocytes with various growth factors and cytokines, such as Ang Ⅱ, which is similar to those induced by pressure or volume load.[20] The characteristic phenotype of cardiomyocyte hypertrophy includes increase of cell volume and protein synthesis,[21] as well as the re-expression of fetal cardiac gene ANF, a feature of hypertrophy and a prognostic indicator of clinical severity.[22] In this study, the findings from surface area, protein content and ANF mRNA expression of the cardiomyocytes indicate that Ang Ⅱ markedly induces cardiomyocyte hypertrophy, and that Evo significantly decreases the elevated surface area, protein content and ANF mRNA expression. Therefore, our results clearly demonstrated that Evo significantly inhibited Ang Ⅱ-induced cardiomyocyte hypertrophy.

Calcium is one potential second messenger

hypothesized to initiate cardiac hypertrophy.[23,24] CaN is a serine/threonine specific phosphatase that is uniquely activated by sustained elevations in intracellular calcium. It is composed of a catalytic subunit referred to CnA, a calcium binding protein referred to CnB.[25] NFAT is dephosphorylated by activation of CaN, and then later translocates to the nucleus where it acts with other transcription factors (eg GATA4) for the activation of downstream target genes to induce cardiac hypertrophy.[26,27] Our results showed that Ang Ⅱ induced cardiomyocyte hypertrophy with the elevated $[Ca^{2+}]_i$, CaN mRNA and CnA protein expressions and Evo could block these increases induced by Ang Ⅱ. It is suggested that the anti-hypertrophic effects of Evo were accompanied with the reduction of $[Ca^{2+}]_i$ concentration and the inhibition CaN signal transduction pathway.

MAPK/ERK signal transduction pathway is regulated by the phosphorylation of the protein kinase to make it active.[10] MAPK/ERK bring the extracellular stimulus signals to the cells to initiate a series of biological changes, such as cell proliferation, differentiation, transformation and apoptosis, and is a critical signal pathway for cardiomyocyte hypertrophy.[28,29] MKP-1 is a double substrate-specific protein kinase, which acts on ERK-2 to make it inactive,[30,31] and thus negatively control MAPK activity. Our results demonstrate that Evo inhibits Ang Ⅱ-induced ERK-2 mRNA expression, upregulates MKP-1 protein expression in a dose-dependent manner. Thus, the anti-cardiomyocyte hypertrophy effects of Evo involve the MAPK/ERK signal transduction pathway.

In recent years, NO has been recognized to be a natural inhibitor of cardiac hypertrophy.[32] NO is endogenously produced by three isoforms of NOS: neuronal isoform (nNOS), inducible isoform (iNOS) and eNOS. Especially, eNOS is constitutively expressed in cardiomyocytes and is dependent on calcium for NO production.[33] Several lines of evidence suggest that eNOS has anti-hypertrophic effects.[34,35] Besides, NO inhibits the L-type Ca^{2+} channel current in cardiomyocytes, and possesses an anti-hypertrophic effect.[36] Our study found that NO content and NOS activity in the cultured medium, as well as eNOS mRNA expression in cardiomyocytes were significantly decreased by Ang Ⅱ, but Evo ameliorated these decreases. Therefore, the increased NO production could be responsible for the anti-hypertrophic effects of Evo.

In conclusion, our results clearly demonstrate that Evo inhibits Ang Ⅱ-induced cardiomyocyte hypertrophy *in vitro*, and this effect is partly due to the promotion of NO production, the reduction of $[Ca^{2+}]_i$ concentration, and the inhibition of CaN and ERK-2 signal transduction pathways.

Conflict of Interest

The authors disclaim no conflict of interest.

Author Contributions

Sun AS conceived and designed the experiments; He N, Gong QH, Zhang F, Zhang JY, Lin SX, Hou HH and Wu Q performed the experiments; Sun AS analyzed the data; He N and Sun AS drafted and revised the manuscript. All of the authors approved the final version.

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