Original Research
Brief Communication

Copper suppression of vascular endothelial growth factor receptor-2 is involved in the regression of cardiomyocyte hypertrophy

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Abstract

Previous studies revealed that copper (Cu)-induced regression of cardiomyocyte hypertrophy is associated with enhanced activity in the vascular endothelial growth factor receptor-1 (VEGFR-1) signaling pathway. The mechanism by which Cu enhances the activity of VEGFR-1 pathway remains to be defined. The present study was undertaken to test the hypothesis that Cu enhances the VEGFR-1 signaling pathway via suppression of the VEGFR-2 signaling pathway. Primary cultures of neonatal rat cardiomyocytes were exposed to phenylephrine (PE) at a final concentration of 100 μM in cultures for 48 h to induce cell hypertrophy. The hypertrophic cardiomyocytes were exposed to copper sulfate at a final concentration of 5 μM Cu in cultures for 24 h. Western blot analysis showed that PE increased the protein levels of both VEGFR-1 and VEGFR-2. Cu supplementation significantly reduced the increase in VEGFR-2, but had no effect on the elevation of VEGFR-1. Real-time polymerase chain reaction analysis found no difference in the mRNA levels between the VEGFR-1 and VEGFR-2 under the conditions defined above. This study thus demonstrated that Cu selectively suppressed PE-elevated VEGFR-2 levels likely via post-translational regulation, leading to the VEGFR-1 signaling pathway becoming dominant and thereby regressing cardiomyocyte hypertrophy.

Keywords: Copper, cardiomyocyte, hypertrophy, VEGF, VEGFR-1, VEGFR-2

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Introduction

Vascular endothelial growth factor (VEGF) is required for hypertrophic growth of cardiomyocytes both *in vitro* and *in vivo* under a diversity of physiological and pathological stimulations.[1–3] We observed that dietary supplementation of physiologically relevant levels of copper (Cu) reverses pressure overload-induced cardiac hypertrophy in mice,[4] and addition of Cu to primary cultures of neonatal rat cardiomyocytes reduces the size of hypertrophic cells induced by phenylephrine (PE).[5,6] Under either *in vivo* or *in vitro* conditions, VEGF is essential for the regression of cardiomyocyte hypertrophy.[4–6] Therefore, VEGF is required for both hypertrophic growth of cardiomyocytes and reversal of cardiomyocyte hypertrophy.

What is the mechanistic understanding of the VEGF requirement for both the hypertrophic growth and the reversal of hypertrophy? In cardiomyocytes, VEGF receptor-2 (VEGFR-2 or kinase insert domain receptor [KDR]) is a major mediator for the action of VEGF. Through interaction with VEGF, the activation of VEGFR-2 initiates a cascade of responses leading to the activation of signaling pathways involving Akt-1, PKC, and ERK1/2,[7–10] which in turn activate the hypertrophic growth machinery.[11–13] On the other hand, VEGFR-1 (or FMS-like tyrosine kinase-1 [Flt-1]) exists in cardiomyocytes and also interacts with VEGF. The activation of VEGFR-1 triggers a cascade of responses leading to the activation of cyclic guanosine monophosphate-dependent protein kinase-1 (PKG-1), which in turn activates the regression pathway in hypertrophic cardiomyocytes, resulting in the reversal of cardiac hypertrophy.[6,14–17]

Studies in primary cultures of neonatal rat cardiomyocytes revealed that the densities of both VEGFR-1 and VEGFR-2 are significantly increased in response to PE stimulation.[6] However, the cells become hypertrophic, suggesting the dominant role of VEGFR-2. Addition of physiologically relevant levels of Cu (5 μM in cultures) suppresses the increase in the VEGFR-2 density, but does not affect the VEGFR-1 density, leading to dominance of the VEGFR-1/PKG-1 pathway.

How does Cu cause the switch from VEGFR-2 to VEGFR-1 dominance? We hypothesize that differential

regulation of the levels of VEGFR-1 and VEGFR-2 protein by Cu would be responsible for the observed switch from the VEGFR-2 to the VEGFR-1 dominant signaling pathway. In the present study, we used primary cultures of neonatal rat cardiomyocytes to examine the effect of Cu on the protein levels of VEGFR-1 and VEGFR-2 in PE-induced hypertrophic cardiomyocytes. Further analysis involved real-time polymerase chain reaction (RT-PCR) measurement of mRNAs for VEGFR-1 and VEGFR-2 in an attempt to define whether the observed difference in the protein levels results from differential regulation of gene transcription.

Materials and methods

Cell culture and treatment

Primary cultures of neonatal rat cardiomyocytes were established according to a procedure published previously,[18] with some modifications. Briefly, the hearts of one- to three-day-old Sprague–Dawley rats were cut into 1-mm pieces and digested with collagenase II (GIBCO, USA) and trypsin (TN, GIBCO, USA) for 5 min, repeating three to five times. The digests were resuspended in H-DMEM (GIBCO, USA) supplemented with antibiotics (GIBCO, USA) and 10% fetal bovine serum (FBS, Hyclone). Fibroblasts were removed by preincubation for 1 h at 37°C. The cardiomyocytes in suspension were collected and seeded in six-well plate containing H-DMEM supplemented with 10% FBS, BrdU (Roche, USA) and antibiotics for 24 h before subsequent experiments. This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication NO. 85-23, revised 1996).The animal procedure was approved by the Institutional Animal Care and Use Committee of Sichuan University West China Hospital.

Experimental procedure

The model of cardiomyocyte hypotrophy was established according to a previous publication.[19] Briefly, primary cultures of neonatal rat cardiomyocytes were treated with PE (Sigma–Aldrich) at a final concentration of 100 μM for 48 h in serum-free media, and then copper sulphate ($CuSO_4$, Sigma–Aldrich) was added directly into the serum-free cultures at a final concentration of 5 μM for an additional 24 h. PE and $CuSO_4$ were dissolved in deionized water and sterilized by filter before they were added to the cells. At the end of the treatment, the cells were collected by trypsinization, suspended in Phosphate buffered saline buffer, and counted using a hemocytometer. Protein content was determined using the Bradford method (Bio-Rad, Hercules, CA) and normalized to cell number. VEGFR-1 and VEGFR-2 proteins were analyzed by Western blotting and their mRNA levels were determined by RT-PCR.

Immunocytochemistry

To examine morphological changes of cardiomyocytes in cultures after PE and Cu treatment, cells were cultured in a six-well plate and treated as described in the experimental procedure. At the end of the respective treatment, cells were fixed in 4% paraformaldehyde in PBS for 40 min. The culture chamber was incubated with monoclonal anti-α sarcomeric actin antibody (Santa Cruz, USA) followed by incubation with Fluorescein isothiocyanate-conjugated goat anti-mouse IgM.[20] Fluorescence was visualized by a confocal microscope (Nikon A1, Japan). Images were acquired using the NIS-Elements software.

Western blotting analysis of VEGFR-1 and VEGFR-2

The protein levels of VEGFR-1 and VEGFR-2 were determined by Western blotting. Cells scraped in PBS were washed three times and lysed using 1% sodium dodecyl sulfate solution. Protein samples were mixed with 5× loading buffer, boiled for 10 min at 100°C and cooled. Equal amounts of protein (50–100 μg) from each sample were separated by 6% SDS-Polyacrylamide gel electrophoresis. Proteins were then transferred to a polyvinylidene fluoride membrane (Bio-Rad, USA). Transferred proteins were blocked with 5% non-fat dry milk in Tris-HCl buffer solution containing Tris-HCl (50 mM), NaCl (150 mM), and Tween-20 (0.1%) (TBS-T) for 1 h at room temperature. The blots were incubated with respective primary antibodies (anti-VEGFR-1, anti-VEGFR-2, Abcam, USA) in blocking solution according to the vender's recommendation. After incubation, the blots were washed with TBS-T six times for 5 min each followed by incubation with appropriate secondary antibody for 2 h. After washing six times (5 min each), target proteins were visualized using chemiluminescence (Bio-Rad, USA) and analyzed by densitometry using Image Pro-Plus Software.

Quantitative RT-PCR

Total RNA was extracted from cardiomyocytes with TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. RNA concentration was quantified with a Gene Quant pro (Amersham Biosciences, GE Healthcare, USA). Complementary DNAs (cDNAs) were synthesized with a SYBR Premix Ex Taq (TaKaRa, Japan) in MJ Mini personal thermal Cycler (Bio-Rad, USA). The amount of cDNA corresponding to 100 ng of RNA was amplified using a SYBR green PCR kit (Applied Biosystems) with the primers for rat *β*-MHC (beta-myosin heavy chain), α-SA (skeletal alpha-actin), ANP (atrial natriuretic peptide), VEGFR-1, VEGFR-2, or RPS 18 (Ribosomal protein 18 S). The primer sequences (Table 1) were designed using the software offered by Takara. RT-PCR reactions were performed, recorded, and analyzed by the iCycler.

Statistics

Data were obtained from three separate experiments and presented as mean values ± SEM. One-way analysis of variance was used for initial analysis and Student–Newman–Keuls was employed for comparison among multiple groups, and differences among treatments were consider significant at $P < 0.05$.

Results

Treatment of primary cultures of neonatal rat cardiomyocytes with PE at a final concentration of 100 μM in cultures for 48 h increased the size of the cardiomyocytes and

Table 1 Primer sequences used for quantitative RT-PCR analysis

β-MHC	5′-TTGGCACGGACTGCGTCATC-3′	(Forward)
	5′-GAGCCTCCAGAGTTTGCTGAAGGA-3′	(Reverse)
α-SA	5′-TCGCGACCTTACTGACTACCTG-3′	(Forward)
	5′-GCTTCTCTTTGATGTCGCGC-3′	(Reverse)
ANP	5′-ATCTGATGGATTTCAAGAACC-3′	(Forward)
	5′-CTCTGAGACGGGTTGACTTC-3′	(Reverse)
VEGFR-1	5′-CTCGTTAGAGATTTGGAAGCGC-3′	(Forward)
	5′-GCAGGGACACCTCTAGCTTGAC′	(Reverse)
VEGFR-2	5′-ACAGTTCCCAGAGTGGTTGG-3′	(Forward)
	5′-GTCACTGACAGAGGCGATGA-3′	(Reverse)
RPS18	5′-CCTTCGCTATCACTGCCATT-3′	(Forward)
	5′-TGGCCAGAACCTGGCTATAC-3′	(Reverse)

β-MHC: beta-myosin heavy chain; α-SA: skeletal alpha-actin; ANP: atrial natriuretic peptide; VEGFR: vascular endothelial growth factor receptor; RPS: ribosomal protein.

significantly increased the protein content. Biomarkers for cardiomyocyte hypertrophy including ANP, β-MHC, and α-SA were all significantly increased, as shown in Figure 1. Under the same conditions, the protein levels of VEGFR-1 and VEGFR-2, as determined by Western blotting analysis, were significantly increased, as shown in Figure 2.

Addition of $CuSO_4$ at a final concentration of 5 μM Cu in cultures for an additional 24 h in the 48 h PE-treated cardiomyocytes resulted in a reduction in cell size and the protein content of the cells, and the reversed elevation of hypertrophic biomarkers (Figure 1). Under this experimental condition, the PE-induced elevation in the level of VEGFR-2 protein was reduced to the level equal to untreated controls (Figure 2). On the other hand, the PE-elevated level of VEGFR-1 protein was not altered by the addition of Cu (Figure 2).

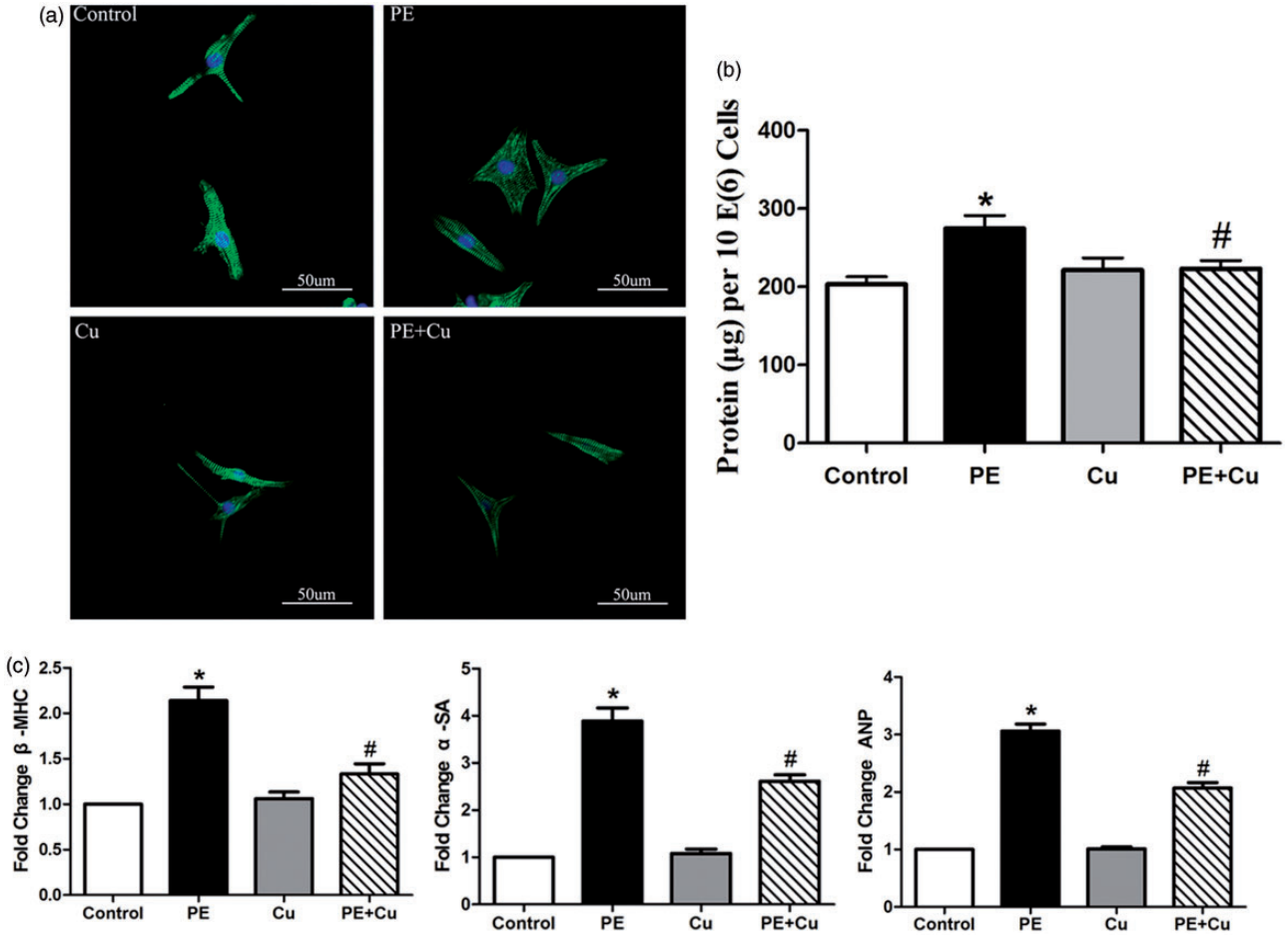

Figure 1 PE-induced cell hypertrophy and its regression by Cu in primary cultures of neonatal rat cardiomyocytes. Cells were treated with 100 μM PE for 48 h in serum-free media before a treatment with 5 μM $CuSO_4$ for additional 24 h in the presence of PE. (a) Representative fluorescence microscopic images of cellular morphology by staining the cells with anti-α-sarcomeric actin antibody in primary cultures of neonatal rat cardiomyocytes. *Control* (non-treated and incubated for 72 h), *PE* (PE treated for 48 + 24 h), *Cu* (Cu treatment during the last 24 h), and *PE + Cu* (PE treated for 48 h followed by Cu for 24 h) 400 ×. (b) Estimation of cardiomyocyte hypertrophy by changes in total protein concentration, normalized by cell number. (c) Changes in the expression of *β-MHC*, *α-SA*, and *ANP*, measured by RT-PCR, in cells after treatment with PE and Cu. Each group of data was obtained from three independent experiments, and each experiment contains triplicate samples for each treatment. Values are means ± SEM. * or # significantly different from control group and # significantly different from PE-treated group ($P < 0.05$). (A color version of this figure is available in the online journal.)
PE: phenylephrine.

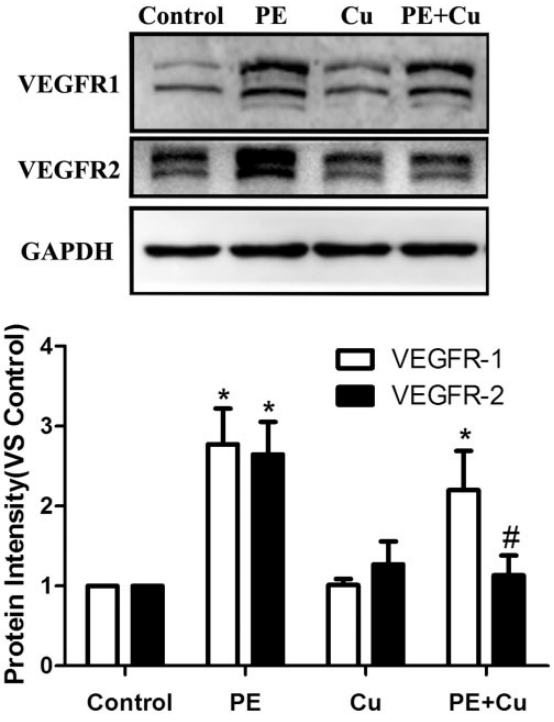

Figure 2 Western blot analysis of changes in VEGFR protein levels induced by PE and Cu treatment in primary cultures of neonatal rat cardiomyocytes. The treatment protocol was as the same as presented in Figure 1, and the cells lysed were subjected to Western blot analysis to determine changes of VEGFR-1 and VEGFR-2.The Western blot procedure was repeated six times with new set of samples obtained from separate experiments. Semi-quantitative analyses based on the density changes of each protein on the blot were obtained from six independent blots. The comparison was done in relation to controls. Values are means ± SEM. * significantly different from control group, # significantly differently from PE-treated group ($P < 0.05$).
PE: phenylephrine; VEGFR: vascular endothelial growth factor receptor; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

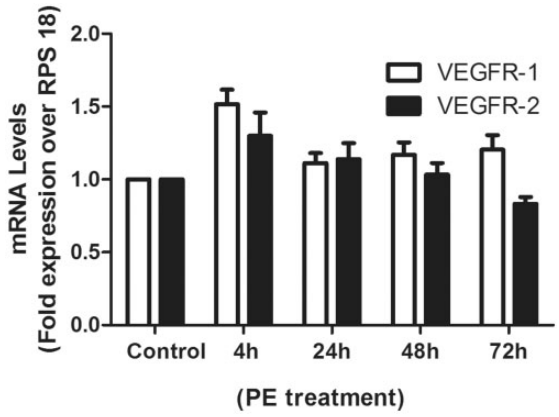

Figure 3 The effect of PE treatment on the levels of mRNAs for VEGFR-1 and VEGFR-2. Cells were treated with 100 μM PE in serum-free media. At the time of four, 24, 48, or 72 h post-PE treatment, the cells were harvested for mRNA analysis. The comparison was done in relation to controls. Each data point was obtained from three independent experiments and each experiment contains triplicate samples for each treatment. Values are means ± SEM.
VEGFR: vascular endothelial growth factor receptor; PE: phenylephrine

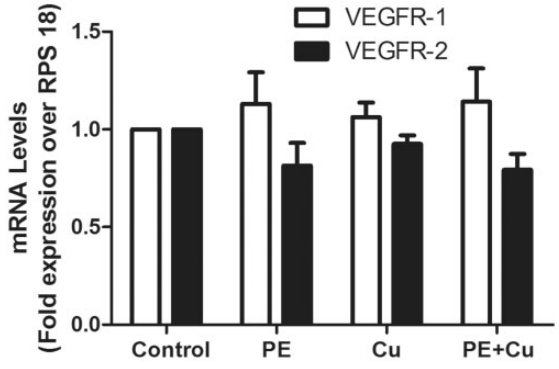

Figure 4 Effects of PE, Cu, or PE plus Cu on the levels of mRNAs for VEGFR-1 and VEGFR-2 in cardiomyocytes. The treatment protocol was as the same as presented in Figure 1. The comparison was done in relation to controls. Each data point was obtained from four independent experiments and each experiment contains triplicate samples for each treatment. Values are means ± SEM.
VEGFR: vascular endothelial growth factor receptor; PE: phenylephrine

To define whether or not the changes in the levels of VEGFR-1 and VEGFR-2 protein were related to changes in gene expression, RT-PCR analysis of the RNA isolated from cardiomyocytes under different treatment conditions was performed. The mRNA levels for VEGFR-1 or VEGFR-2 were not significantly changed in response to PE treatment at the times examined (4, 24, 48, or 72 h post PE treatment), as shown in Figure 3. The additional Cu treatment did not significantly change the mRNA levels for VEGFR-1 or VEGFR-2 either (Figure 4).

Discussion

The observation that VEGF is required for both hypertrophic growth and the reversal of hypertrophy in cardiomyocytes[4,5] promotes us to search for the mechanistic understanding. Previous studies have identified the role of VEGFR-2 in promoting hypertrophic growth of cardiomyocytes[7–13] and that of VEGFR-1 in the regression of cardiac hypertrophy.[6,14–17] These two receptors are both VEGF-dependent, but trigger opposite responses upon their activation by VEGF in cardiomyocytes.[17] In primary cultures of neonatal rat cardiomyocytes, PE treatment stimulates the hypertrophic growth of these cells. However, PE-treatment not only increased the density of VEGFR-2, but also enhanced the density of VEGFR-1 in the cardiomyocytes in cultures.[6] It is plausible that when VEGFR-2 is stimulated, the counteraction of VEGFR-1 is also triggered to balance the overgrowth. The dominant role of VEGFR-2 under the PE treatment condition indicates an insufficient counteraction of VEGFR-1 in cardiomyocytes. However, the addition of physiologically relevant concentrations of Cu makes the counteraction of VEGFR-1 become dominant.[6] The present study revealed that this switch takes place because Cu reduces the elevated VEGFR-2 in the hypertrophic cardiomyocytes.

If Cu is a critical element in the regulation of the balance between VEGFR-2 and VEGFR-1 signaling pathways, changes in Cu status under different stress conditions could affect the ultimate physiological and pathological

outcome. We have observed that pressure overload causes Cu loss in the heart along with the progression of cardiac hypertrophy and Cu supplementation reverses cardiac hypertrophy and recovers the defected heart function.[4] In the PE-treated primary cultures of neonatal rat cardiomyocytes, homocysteine levels increase along with a decrease in Cu concentrations.[21] Further analysis found that homocysteine forms complexes with Cu, limiting the availability of Cu to Cu-dependent proteins as well as causing an efflux of Cu from the cells.[21,22] Addition of Cu to the PE-treated cultures of cardiomyocytes replenishes the Cu pool and refreshes Cu availability, leading to the relief of the suppressed function of Cu-dependent proteins.[21,22] Therefore, under the condition of PE treatment without Cu addition, the limited availability of Cu would be associated with the dominant action of VEGFR-2 over VEGFR-1 in the hypertrophic cardiomyocytes.

The switch from VEGFR-2 dominant to VEGFR-1 dominant is Cu-dependent. This was demonstrated in our previous studies[6] and further confirmed in the present study. Although the increase in the protein levels of VEGFR-2 was related to the dominant role of this signaling pathway, the depression of its domination by the addition of Cu was not due to a decreased production of this protein, as reflected by the fact that the mRNA levels for VEGFR-2 or VEGFR-1 were not altered by either PE treatment or the additional Cu treatment. Therefore, Cu effect on the differential regulation of VEGFR-2 and VEGFR-1 signaling pathways would be mediated by its possible post-translational action on these proteins.

VEGFR-1 and VEGFR-2 are members of receptor tyrosine kinases family (RTKs). In general, RTKs undergo tyrosine phosphorylation-dependent ubiquitination and degradation involving c-Cbl ubiquitin E3 ligase family proteins.[23] An early study has confirmed that c-Cbl could regulate the VEGF-induced endocytosis and degradation of VEGFR-1 through binding to the tyrosine 1333 in VEGFR-1.[24] However, unlike other RTKs, VEGFR-2 ubiquitination and degradation is not mediated by c-Cbl,[25] but mainly regulated through β-Trcp 1, another member of ubiquitin E3 ligase.[26] Coincidentally, some Cu binding proteins possess E3 ubiquitin ligase activity, such as X-linked inhibitor of apoptosis protein (XIAP), which can target various proteins for ubiquitination and degradation, including some members of RTKs.[27–30] Therefore, we speculate that Cu differentially regulates the levels of VEGFR-1 and VEGFR-2 proteins through some Cu binding proteins, which directly or indirectly regulate the ubiquitination and degradation of VEGFR-1 and VEGFR-2 proteins. This will be our future undertaking in order to provide further mechanistic understanding.

In conclusion, the present study provides further understanding of Cu reversal of cardiomyocyte hypertrophy. Cu depression of VEGFR-2 signaling pathway plays a critical role in the activation of VEGFR-1/PKG-1 signaling pathways, leading to regression of cardiac hypertrophy. The differential regulation of the VEGFR-2 and VEGFR-1 signaling pathways by Cu would take place at the post-translational events, rather than at the transcriptional level.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; TW, RL, CL, and MS carried out the experiments; YJK and TW wrote the manuscript. TW and RL made equal contributions to this study.

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