Original Research

Vascular endothelial growth factor recovers suppressed cytochrome c oxidase activity by restoring copper availability in hypertrophic cardiomyocytes

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Abstract

Cardiomyocyte hypertrophy induced by phenylepherine (PE) is accompanied by depression of cytochrome c oxidase (COX) activity. Vascular endothelial growth factor (VEGF) recovers the suppressed COX activity and reverses cardiomyocyte hypertrophy. Because PE causes intracellular copper (Cu) depletion and COX activity is Cu-dependent, the present study was undertaken to test the hypothesis that VEGF recovers suppressed COX activity by restoring Cu availability. Primary cultures of neonatal rat cardiomyocytes were treated with PE at a final concentration of 100 μmol/L in cultures for 48 h to induce cell hypertrophy. The hypertrophic cardiomyocytes were exposed to VEGF at a final concentration of 20 ng/mL in cultures for 24 h. Atomic absorption spectrometry analysis revealed that VEGF restored PE-depleted Cu concentrations in hypertrophic cardiomyocytes along with the recovery of COX activity. Western blot analysis showed that protein contents of COX subunit COX-IV and Cu chaperones for COX (COX17, COX11, and SCO2) were decreased in response to PE treatment, and recovered after VEGF treatment. In addition, VEGF treatment suppressed PE-induced accumulation of reactive oxygen species (ROS) and the relevant elevation of homocysteine, which has been shown to form complexes with Cu to restrict Cu availability. This study thus demonstrates that VEGF recovers PE-suppressed COX activity by restoring Cu availability and VEGF suppression of ROS accumulation and homocysteine elevation would contribute to the increased Cu availability.

Keywords: Cardiomyocyte, cytochrome c oxidase, homocysteine, hypertrophy, vascular endothelial growth factor

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Introduction

Dietary copper (Cu) supplementation reverses pressure overload-induced cardiac hypertrophy in mouse model.[1] Cu concentrations are reduced in the hypertrophic heart along with depression of Cu-dependent cytochrome c oxidase (COX) activity and suppressed angiogenesis. Therefore, in the Cu-induced regression of cardiac hypertrophy, enhanced production of vascular endothelial growth factor (VEGF) that is required for angiogenesis and recovery of COX activity are essential.[1–4] We observed that both Cu supplementation and VEGF addition recover the suppressed COX activity and reverse cardiomyocyte hypertrophy.[1,4]

COX is an important enzyme in sustaining mitochondrial function. It is the terminal enzyme of respiratory chain and is in charge of the reduction of oxygen molecule to water.[5–7] COX consists of 13 subunits, three of them (COX-I, -II, -III) are encoded by the mitochondrial DNA, the others are encoded by the nuclear DNA.[8–11] There are two Cu-binding sites in COX and Cu deficiency suppresses COX activity.[12–16] Cu chaperones for COX, including COX assembly homolog 17 (COX17) and 11 (COX11), COX-deficient homolog 1 (SCO1) and 2 (SCO2), are responsible for delivering Cu to the Cu active sites Cu_A in COX-II and Cu_B in COX-I subunits of COX.[17–20]

Phenylepherine (PE) treatment increases cellular accumulation of reactive oxygen species (ROS),[21] which in turn causes elevation of homocysteine concentrations in primary cultures of neonatal rat cardiomyocytes.[22] Homocysteine and Cu form complexes, thus reducing intracellular Cu concentrations and limiting Cu availability. This in turn leads to suppression of COX activity.[23–25] Furthermore, Cu chaperones COX17, COX11, SCO2, and COX subunit COX-IV are suppressed under PE treatment. Cu supplementation recovers COX activity and relieves the suppression on Cu chaperones and COX-IV. Inhibition of intracellular homocysteine synthesis results in the same rescuing effect on COX activity, Cu chaperones, and COX-IV as does Cu supplementation.[23] Therefore, PE-induced elevation of

homocysteine restricts Cu availability through the formation of Cu–homocysteine complexes, leading to suppression of Cu-dependent proteins including COX and Cu chaperones.[23–25]

Therefore, the present study was undertaken to test the hypothesis that VEGF suppresses the accumulation of ROS and homocysteine production to restore intracellular Cu availability, ensuring Cu trafficking and transferring to COX, which leads to the recovery of COX activity.

Materials and methods

Cell culture

Primary cultures of neonatal rat cardiomyocytes were established according to a procedure published previously,[26] with some modifications. Briefly, the hearts of 1- to 3-day-old Sprague–Dawley rats were washed with H-DMEM (Gibco, USA), cut into 1 mm, and digested with collagenase II (Gibco) and trypsin (TN, GIBCO, USA) for 5 min in 37°C, repeating 5–7 times. The digests were suspended in H-DMEM supplemented with antibiotics (Gibco) and 10% fetal bovine serum (FBS, Hyclone). Fibroblasts were removed by pre-incubation for 30 min in the 37°C incubator. Cardiomyocytes in the suspension were collected and seeded in T25-culture flask containing H-DMEM supplemented with 10% FBS, BrdU (Roche), and antibiotics for 24 h before subsequent experiments. This investigation 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.

Experimental procedure

The model of cardiomyocyte hypotrophy was established according to a previous publication.[27] Briefly, primary cultures of neonatal rat cardiomyocytes were treated with phenylephrine (PE, Sigma–Aldrich) at a final concentration of 100 μmol/L for 48 h in serum-free media, and then VEGF (Gibco) was added directly into the serum-free cultures at a final concentration of 20 ng/mL for additional 24 h. At the end of the treatment, the cells were collected by trypsinization, suspended in phosphate-buffered saline (PBS) buffer, and counted using a hemocytometer. Protein content was measured using the BCA kit (Thermo Fisher, USA). Cell hypertrophy was confirmed by the increase in cell size and cellular protein content, as described previously.[3,4]

Table 1 Effect of VEGF on COX activity in PE-treated cardiomyocytes[a]

COX activity μmol cyto c/mg protein/min	Control	PE	VEGF	PE + VEGF
	5.16	3.62*	5.27	4.58
SD	0.25	0.19	0.17	0.07

[a]Each data point was obtained from three independent experiments.
*Significantly different from control group ($P < 0.05$).

Measurement of COX activity

Cells were trypsinized, washed, and suspended with PBS. The lysate was added directly into a reaction tube and incubated for 30 min. The protein was collected by centrifugation at 13,000 *g* for 10 min at 4°C. COX activity was assayed using a COX assay kit following the provided instruction (Genmed, China). The data were normalized by cellular protein concentrations.

Measurement of ROS

ROS accumulation in the cell was measured using CellROX® Deep Red reagent from Invitrogen. Cells were incubated at 37°C for 30 min in culture media containing 3 μmol/L CellROX® Deep Red reagent. The nucleus was counterstain with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). Cells were then washed twice in PBS, the fluorescence with 640/665 nm was immediately measured using confocal microscope (Olympus, Japan). Images were collected by fluorescence microscopy and fluorescence intensity was analyzed using the Image pro plus and normalized with cell number.

Determination of Cu concentrations

Intracellular Cu concentrations were determined with graphite furnace atomic absorption spectrophotometer (AAS). Cells were collected by trysinization, washed three times with phosphate buffer solution, and centrifuged at 500 *g* for 5 min. The precipitation was lysed in 1% sodium dodecyl sulfate (SDS) with ultrasonication. Then the lysis was centrifuged at 13,000 *g* at 4°C for 10 min. The supernatant was collected and dissolved in five volumes of HNO_3 for two days. Finally, the prepared samples were dissolved in four volumes of deionized water, and then subjected to AAS analysis.

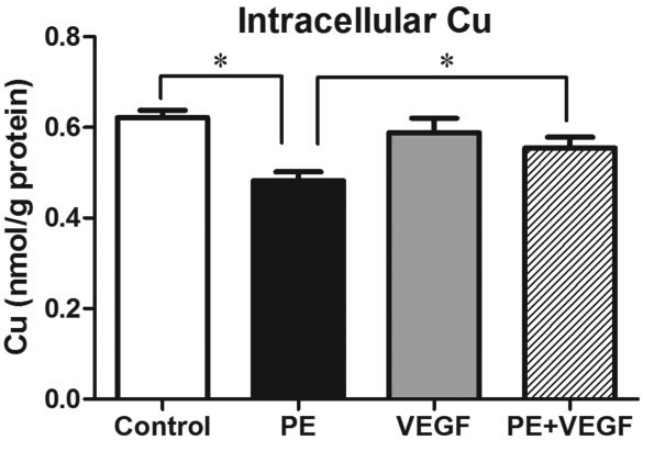

Figure 1 Effect of VEGF on intracellular Cu concentrations in PE-treated cardiomyocytes. Cells were treated with 100 μmol/L PE for 48 h in serum-free media before the treatment with 20 ng/mL VEGF for additional 24 h in the presence of PE. The cell lysate was subjected to nitrification before the measurement with graphite furnace atomic absorption spectrophotometer (AAS). Each data point was obtained from 12 independent experiments. Values are means ± SEM.
*Significantly different from each other ($P < 0.05$)

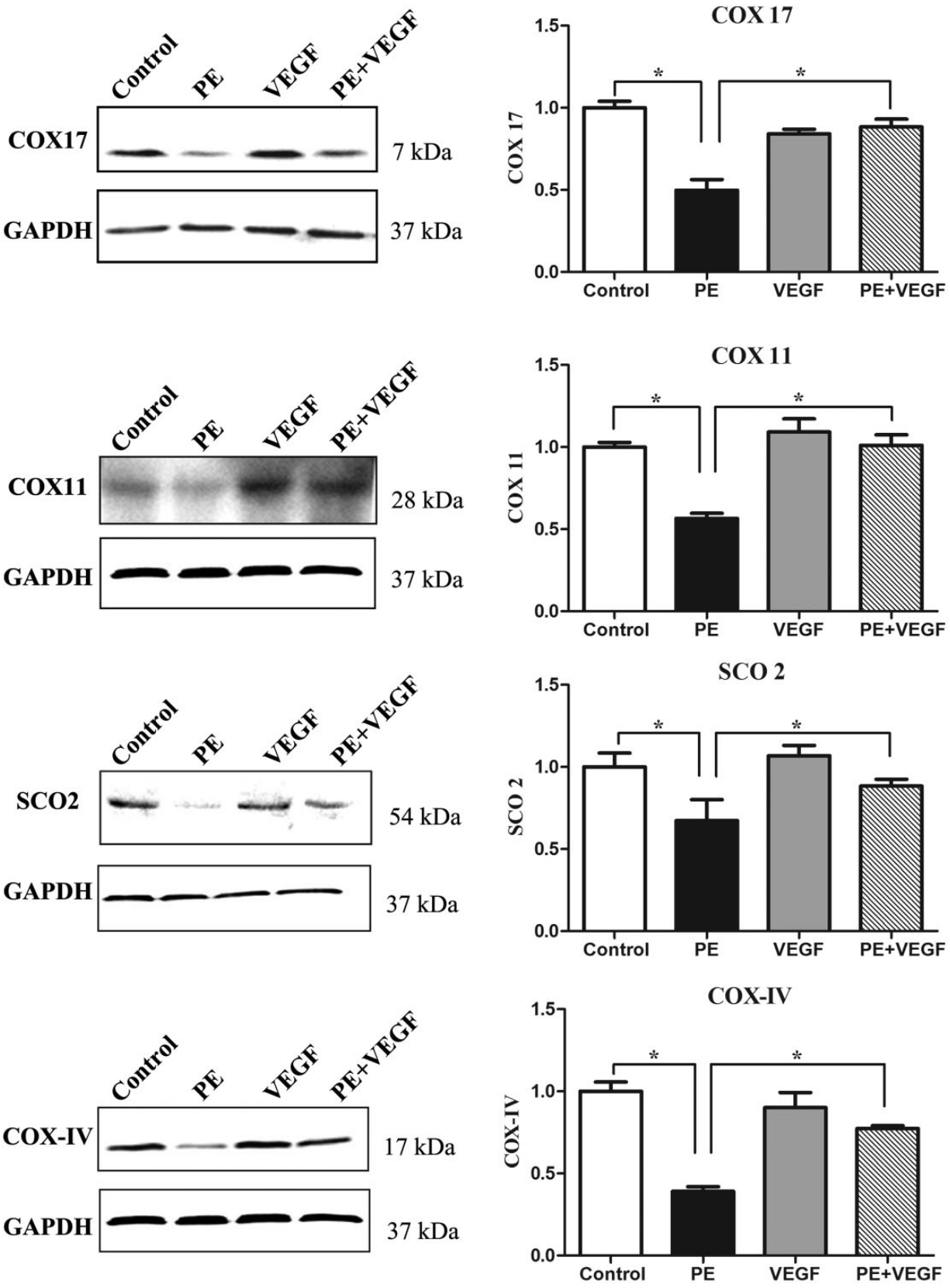

Figure 2 Western blot analysis of COX17, COX11, SCO2, and COX-IV protein levels. The Western blotting procedure was repeated three times with new set of samples obtained from separate experiments. Semiquantitative analyses based on the density changes of each protein on the blot were obtained from three independent blots. The comparison was done in relation to controls. Values are means ± SEM. *Significantly different from each other ($P < 0.05$)

Measurement of homocysteine concentrations

Extracellular homocysteine was measured by using HPLC as described before.[28] HPLC analysis was performed using Waters Millenium system (Waters 600) with a Waters 474 Fluorescent Detector and a Supelcosil LC 18 DB analytical column (250 mm × 64.6 mm, 3 μmol/L particle size). The temperature inside the column was maintained at 25°C during elution. The sample was prepared as follows: an aliquot of 90 μL cell culture media and 10 μL reducing agent (10% tris [2-carboxyethyl] phosphine hydrochloride, TCEP, Sigma) were mixed and incubated for 30 min at 4°C. The precipitation of proteins was discarded by addition of 100 μL methanol and subsequent centrifugation at 20,000 *g* for 10 min. To 160 μL of supernatant, the following were added orderly: 250 μL of borate buffer (0.125 mol/L) containing EDTA (4 mmol/L) adjusted to pH 9.5 and 10 μL of NaOH (1.55 mol/L) and 10 μL of 7-fluorobenzofurazan-4-sulfonic ammonium salt (SBD-F, 10 mg/mL solution in 0.125 mol/L borate buffer, pH 9.5). The mixture was incubated for 60 min at 60°C and subjected to HPLC separation and analysis. The HPLC was performed as follows: mobile phase was 0.05 mol/L KH_2PO_4, adjusted pH to 2.1 with orthophosphoric acid and 7% acetonitrile; flow-rate was 1.0 mL/min; and total elution time was 20 min. Fluorescent was monitored at 515 nm and excitation wavelength was 385 nm. Calibration was based on external standard using homocysteine diluted in distilled water and peak area was used for calculation of concentrations.

Western blotting of COX17, COX11, SCO2, and COX-IV

The protein contents of COX17, COX11, SCO2, and COX-IV were determined by Western blot. Cells scraped in PBS were washed three times, and lysed in 1% SDS solution after ultrasonication. The cell lysate was collected by centrifugation at 13,000 *g* for 10 min at 4°C. Protein samples were mixed with 5X loading buffer, boiled for 10 min at 100°C, and cooled. The samples were stored at −20°C. Equal amounts of protein (50–100 mg) from each sample were separated by 12% SDS-PAGE. Proteins were then electrophoretically transferred to a polyvinylidene fluoride membrane (Bio-Rad, USA). Transferred proteins were blocked with 5% non-fat dry milk in Tris-HCl buffer containing Tris-HCl (50 mmol/L), NaCl (150 mmol/L), and Tween-20 (0.1%) (TBS-T) for 1 h at room temperature. The blots were then incubated with respective primary antibodies (anti-COX17, anti-COX11, and anti-COX-IV, Santa Cruz, USA; anti-SCO2, Abcam, USA) in blocking solution following the manufacture's recommendation. After incubation, the blots were washed with TBS-T six times for 5 min each and incubated for 2 h with appropriate secondary antibody. After washing six times (5 min each), target proteins were visualized using chemiluminescence (Bio-rad) and analyzed by densitometry using a Quantity One Software.

Statistical analysis

Data were obtained from three separate experiments and expressed as means ± standard error of the mean (SEM). A multiple factorial design was applied to this study. After a significant interaction was detected by the analysis of variance (ANOVA), the significance of the main effects was further determined by *F*-test. The level of significance was considered when $P < 0.05$.

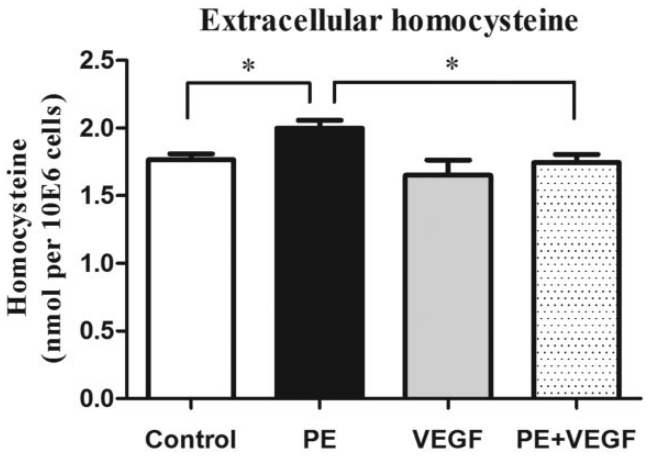

Figure 3 Effect of VEGF on homocysteine levels in cardiomyocytes. Homocysteine levels in culture media were measured by HPLC. Each data point was obtained from seven independent experiments. Values are means ± SEM. *Significantly different from each other ($P < 0.05$)

Results

Recovery by VEGF of suppressed COX activity and Cu concentrations

The treatment of primary cultures of neonatal rat cardiomyocytes with 100 μmol/L PE for 48 h resulted in cell hypertrophy (data not shown). The hypertrophic cardiomyocytes were than treated with VEGF at a final concentration of 20 ng/mL for 24 h. COX activity was depressed in the cells treated with PE for 72 h, and this depression was reversed after VEGF treatment for 24 h (Table 1). Cu concentrations in the cells treated with PE only were significantly decreased, and this decrease was also reversed after VEGF treatment (Figure 1). VEGF treatment alone affected neither COX activity nor cellular Cu concentrations (Table 1 and Figure 1).

VEGF reversal of PE-induced depression of protein levels of COX subunit COX-IV, and Cu chaperones COX17, COX11, and SCO2

We recently observed that changes in intracellular Cu concentrations affect the protein levels of Cu chaperones.[23,29] To determine whether VEGF affects Cu chaperones in the PE-treated cardiomyocytes, we measured the protein levels of COX17, COX11, SCO2, and COX-IV. The data presented in Figure 2 showed that PE reduced the protein levels of the measured Cu chaperones and COX-IV. Addition of VEGF to the PE-treated cardiomyocyte cultures during the last 24 h completely rescued the suppression of all of the Cu chaperones and COX-IV.

VEGF suppression of PE-induced elevation of homocysteine in cardiomyocytes

We measured intracellular homocysteine levels to determine whether or not VEGF recovery of Cu availability in

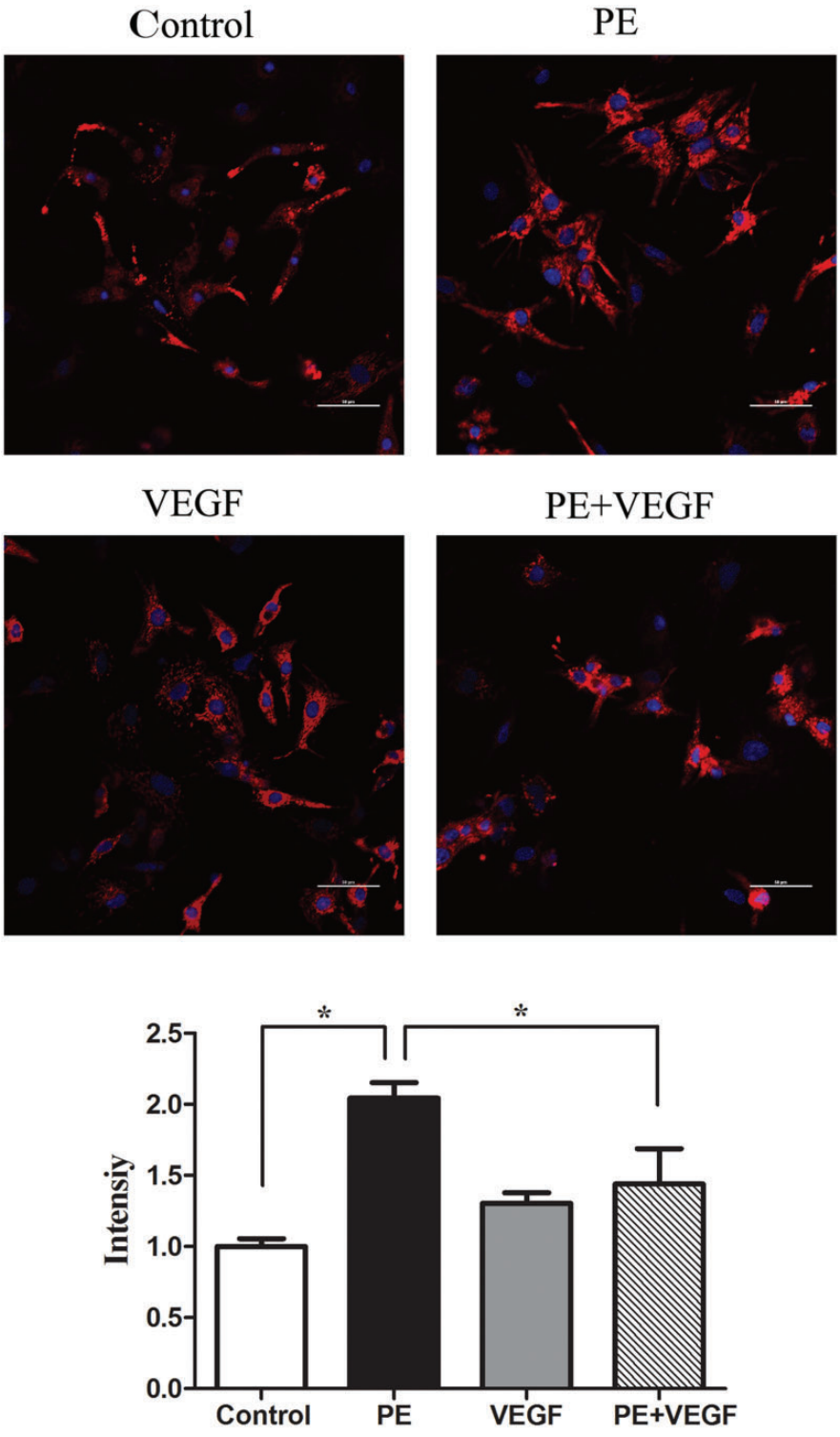

Figure 4 Effect of VEGF on ROS accumulation in PE-treated cardiomyocytes. Live cell staining was performed in cardiomyocytes using ROS sensor CellROX® Deep Red reagent (red) and nuclear marker (blue) with a confocal microscope. Scale bar = 50 μm. Each data point was obtained from three independent experiments. Semiquantitative analyses based on the fluorescence intensity of each group were obtained from three independent fields from each slide, and three slides from each treatment. The comparison was done in relation to controls. Values are means ± SEM. *Significantly different from each other ($P < 0.05$). (A color version of this figure is available in the online journal)

PE-treated cardiomyocytes is mediated by suppression of homocysteine production. As shown in Figure 3, PE treatment significantly increased homocysteine concentrations and this increase was suppressed by VEGF treatment.

VEGF inhibition of PE-induced accumulation of ROS in cardiomyocytes

ROS accumulation is a major reason for increased homocysteine production, therefore, we measured the changes in

intracellular ROS levels. As shown in Figure 4, PE treatment significantly increased ROS accumulation and VEGF treatment suppressed this accumulation (Figure 4).

Discussion

Regression of pressure overload-induced cardiac hypertrophy by Cu supplementation requires the recovery of depressed COX activity and VEGF production.[1–4] The data obtained from this study show that VEGF relieved the suppression of COX activity, which was accompanied by replenishment of Cu levels. VEGF also recovered PE-depressed Cu chaperones for COX including COX17, COX11 and SCO2, and COX subunit COX-IV. Importantly, VEGF suppressed the accumulation of ROS and the elevation of homocysteine levels in response to PE treatment. This provides a critical insight into the mechanism by which VEGF restores the depressed COX activity.

Gene silencing of COX-I subunit of COX results in an inhibition of COX activity, and blocks Cu regression of cardiomyocyte hypertrophy.[4] VEGF-induced regression of cardiomyocyte hypertrophy is also abolished by COX-I gene silencing.[4] Therefore, it is important to understand the link between VEGF and Cu replenishment and its relation to COX recovery in hypertrophic cardiomyocytes.

VEGF replenished Cu levels in PE-induced hypertrophic cardiomyocytes, thus recovered COX activity. How does VEGF restore the availability of Cu? The data obtained indicated that VEGF suppression of ROS accumulation and homocysteine elevation was most likely responsible for Cu restoration. PE treatment causes accumulation of ROS[21] and oxidative inhibition of cystathionine β-synthase leads to increase in intracellular homocysteine.[22] Homocysteine restricts the availability of Cu through the formation of Cu–homocysteine complexes.[23,25]

We also observed that VEGF treatment relieved the suppression of Cu chaperone COX17, COX11, and SCO2, and COX subunit COX-IV. These chaperones are required for Cu insertion into COX complex during its assembly process.[14–16] We have observed that suppression of these Cu chaperones and COX-IV was related to diminished Cu availability because either Cu supplementation or inhibition of homocysteine synthesis reversed PE suppression of these Cu chaperones and COX-IV.[23] Therefore, VEGF, through suppressing the elevation of homocysteine levels, restored PE-depressed Cu chaperones and COX-IV, leading to normalization of Cu intracellular trafficking and further ensuring the recovery of COX activity.

The important finding of this study was that VEGF suppressed PE-induced elevation of homocysteine levels, thus restoring the availability of intracellular Cu due to reduced formation of Cu–homocysteine complexes. However, the limitation of this study, as other *in-vitro* studies, is that it needs to be validated *in vivo*. In addition, the use of neonatal rat cardiomyocytes may not be necessarily applied to adult cardiomyocytes because there are potential differences in their responses to VEGF. This and other possible questions will be addressed in our future studies.

In conclusion, the present study addressed the question – how VEGF treatment recovers suppressed COX activity in PE-induced hypertrophic cardiomyocytes. The data obtained demonstrate that VEGF, through suppressing the accumulation of ROS and the elevation of homocysteine levels, restores Cu availability under the condition of PE treatment, thereby ensuring Cu intracellular trafficking and transferring to critical Cu-proteins such as COX, leading to the recovery of COX activity.

Author contributions: All authors participated in the design, interpretation and analysis of the data, and review of the manuscript; MS, XZ, RL, and TW carried out the experiments; YJK and MS wrote the manuscript.

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