

PEDF protects cardiomyocytes by promoting FUNDC1-mediated mitophagy via PEDF-R under hypoxic condition

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Received December 25, 2016; Accepted February 15, 2018

DOI: 10.3892/ijmm.2018.3536

Abstract. Pigment epithelial-derived factor (PEDF) is known to exert diverse physiological activities. Previous studies suggest that hypoxia could induce mitophagy. Astoundingly, under hypoxic condition, we found that PEDF decreased the mitochondrial density of cardiomyocytes. In this study, we evaluated whether PEDF could decrease the mitochondrial density and play a protective role in hypoxic cardiomyocytes via promoting mitophagy. Immunostaining and western blotting were used to analyze mitochondrial density and mitophagy of hypoxic cardiomyocytes. Gas chromatography-mass spectrometry and ELISA were used to analyze levels of palmitic acid and diacylglycerol. Transmission Electron Microscopy was used to detect mitophagy and the mitochondrial density in adult male Sprague-Dawley rat model of acute myocardial infarction. Compared to the control group, we observed that PEDF decreased mitochondrial density through promoting hypoxic cardiomyocyte mitophagy. PEDF increased the levels of palmitic acid and diacylglycerol, and then upregulated the levels of protein kinase C α (PKC- α) and its activation. Furthermore, inhibition of PKC- α by Go6976 could effectively suppress PEDF-induced mitophagy. Besides, we found that PEDF promoted FUNDC1-mediated cardiomyocyte mitophagy via ULK1, which depended on the activation of PKC- α . Finally, we discovered that mitophagy was increased

and mitochondrial density was reduced in adult male Sprague-Dawley rat model of acute myocardial infarction. We concluded that PEDF promotes mitophagy to protect hypoxic cardiomyocytes, through PEDF/PEDF-R/PA/DAG/PKC- α /ULK1/FUNDC1 pathway.

Introduction

Acute myocardial infarction (AMI) is still one of the leading causes of morbidity and mortality although medical and device therapy has been markedly improved (1). AMI causes the dysfunction of cardiomyocytes and induces damage to mitochondria (2). Dysregulated mitochondria can lead to the gross production of reactive oxygen species (ROS) as an inevitable by-product, which could cause damage to cellular DNA and protein, even programmed cell death (3). Therefore, clearing away the damaged mitochondria is essential for mitochondrial quality control and normal cellular functions (4,5).

Pigment epithelial-derived factor (PEDF), a multifunctional glycoprotein (6), protects against hypoxia-induced apoptosis and necroptosis in primary cardiomyocytes (7). In the study of PEDF protecting hypoxic cardiomyocytes, we first observed that the mitochondrial density of cardiomyocytes treated with PEDF was decreased significantly comparing with that under hypoxia only. Previous studies have shown that enhancement of mitophagy or mitochondrial fusion is impacted by the decrease of mitochondrial density (8,9). Studies have linked damaged mitochondria to progression of heart failure and age associated cardiac pathologies (10,11). Recent studies have suggested that mitophagy plays a specific role in eliciting cardioprotective benefits by removing damaged mitochondria (12,13). However, whether PEDF could decrease the mitochondrial density of hypoxic cardiomyocytes via mitophagy and/or mitochondrial fusion and whether this effect is associated with cardioprotection remain unclear.

Recent study revealed adipose triglyceride lipase (ATGL) as a receptor for PEDF in retinal epithelial cells and cardiomyocytes (14,15). Based on the study of Subramanian *et al*, we used PEDF-R for the 80-kDa receptor protein which interacts with PEDF on the cell surface (16). Notari *et al* used a cell-free system to show that this interaction induced lipase activity (14). PEDF-R is the key enzyme of lipid catabolism and catalyzes the lipid lipolysis cascade, generating free fatty acids (FFAs) and

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Key words: pigment epithelial-derived factor, pigment epithelial-derived factor-receptor, palmitic acid, diacylglycerol, protein kinase C α , FUNDC1, mitophagy

diacylglycerol (DAG) (17). In an earlier study we demonstrated that PEDF could increase the level of FFAs in cardiomyocytes after AMI via PEDF-R (15). Although Tan *et al* have reported induction of autophagy by palmitic acid (PA) via DAG-PKC- α pathway (18), the signaling pathway linking PA stimulation to mitophagy in response to PEDF remains to be determined.

The protein kinase C (PKC) family plays a role downstream of many signal transduction pathways (19,20) and PKC- α is the predominant conventional PKC isoform expressed in the mouse, rat, and human heart (21,22). Mitophagy plays a major role in mitochondrial quality control through three pathways, involving Parkin, Nix (also known as BNIP3L) and FUNDC1 (23-25). Particularly, recent studies suggest that ULK1 and SRC have a more specific effect on mitophagy through interacting with its substrate FUNDC1 (24,26). However, it is not clear whether PKC- α could modulate mitophagy or not.

Here, we first found PEDF decreased the mitochondrial density of cardiomyocytes via promoting mitophagy under hypoxic condition. In addition, PEDF-mediated mitophagy was found to serve as a survival mechanism of cardiomyocytes under hypoxic environment. Furthermore, we identified a novel signaling pathway, PEDF/PEDF-R/PA/DAG/PKC- α /ULK1/FUNDC1, associated with PEDF-mediated mitophagy.

Materials and methods

Antibodies and reagents. Anti-LC3B antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The Go6976 and bafilomycin A1 (BAF1) were obtained from Selleck, Inc. (Houston, TX, USA). Anti-phospho-PKC- α antibody was purchased from Millipore (Billerica, MA, USA). Diacylglycerol (DAG) enzyme-linked immunosorbent assay (ELISA) kit was purchased from USCIN, Inc. (Wuhan, China). MitoTracker[®] Red CMXRos was purchased from Invitrogen, Inc. (Paisley, UK). Bromodeoxyuridine (BRDU) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Anti-PKC- α antibody was purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA). Anti-Mfn1, Mfn2, Opa1, Nix, Parkin and ULK1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Alexa Fluor 488 was purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA). Anti-FUNDC1 polyclonal antibody was purchased from Aviva PLC, Inc. (San Diego, CA, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). LDH cytotoxicity assay kit was purchased from Keygen Biotech, Co. (Nanjing, China).

Recombinant lentivirus constructs and viral production. Recombinant lentivirus (LV) was prepared as described previously (27). PEDF overexpression plasmids and the RNAi vector PEDF-R-RNAi_LV of the PEDF-R gene producing PEDF-R shRNA were successfully constructed and then successfully packaged in 293T cells. The concentrated titer of virus suspension was 2×10^{12} TU/l.

Induction of AMI. All the procedures were performed following the guidelines of the Directive 2010/63/EU of the European

Parliament, and have been approved by the Ethics Committee for Animal Experimentation of the Institutions where experiments were carried out. Adult male Sprague-Dawley (SD) rats (200-250 g) were purchased from the Experimental Animal Center of Xuzhou Medical University. Myocardial ischemia was induced by ligation of the left-anterior descending coronary artery (LAD) in anesthetized rats, as described previously (28). The animal models were randomly divided into four groups: normal; normal+PEDF, PEDF-lentivirus was transferred before surgery; AMI (1, 2, 3 and 4 h); AMI+PEDF (1, 2, 3 and 4 h). The rats were anaesthetized with ketamine at 100 mg/kg and xylazine at 10 mg/kg and maintained under anesthesia using isoflurane (1.5-2.0%) mixed with air. During the surgical procedure, the absence of the pedal reflex was used as an indication that a surgical plane of anesthesia was maintained. With the animal lying flat, left thoracotomy was performed through the fourth intercostal space, PEDF-lentivirus (2×10^7 TU) in 20 μ l enhanced infection solution (ENIS, pH 7.4) was delivered with a 20- μ l syringe and 25-gauge needle into the myocardium along LAD. Control animals received an equivalent volume of lentivirus vector in ENIS. The chest cavity was then closed. After reinstallation of spontaneous respiration, animals were extubated and allowed to recover from anesthesia. Buprenorphine was administered at 0.5 mg/kg for postoperative analgesia. In the same way, LAD was ligated with 6-0 silk suture (Ethicon; Johnson and Johnson, Somerville, NJ, USA) after 7 days. Animals were sacrificed with an overdose of sodium pentobarbitone (100 mg/kg, i.v.), and their hearts were harvested at 1, 2, 3 and 4 h after induction of MI for further analysis. Sham-operated rats underwent the same procedure, excluding coronary artery ligation.

Primary cardiomyocyte isolation, culture and infection. Cardiomyocytes were obtained from 1-3-day-old neonatal SD rats as previously described (7,29,30). Briefly, neonatal rats were sacrificed by rapid decapitation and hearts were rapidly removed and placed into dishes on ice, then hearts were dissected and minced into 1 mm³ pieces with sharp scissors, then transferred to a sterile tube. The minced tissue was digested in a phosphate-buffered saline (PBS) solution supplemented with 0.5% trypsin, 0.1% collagenase and 0.02% glucose for 5 min at 37°C. Then cells were incubated for 1 h in the presence of 0.1 mmol/l bromodeoxyuridine to selectively enrich cardiomyocytes. The inclusion of BRDU resulted in inhibition of the growth of cardiac fibroblasts. The resultant cell suspension (10,000-12,000 cells/cm²) was plated onto 48 well culture plate in DMEM/low glucose (HyClone) supplemented with 10% fetal bovine serum and 100 mg/ml penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Hypoxia was achieved by culturing the cells in D-Hank's liquid with glucose deprivation in a tri-gas incubator (Heal Force, Shanghai, China) saturated with 5% CO₂/1% O₂ at 37°C for the indicated times.

Western blotting. For western blot analysis the cells were solubilized in lysis buffer [100 mmol/l Tris-HCl, 4% sodium dodecyl sulfate (SDS), 20% glycerine, 200 mmol/l DTT and protease inhibitors, pH 6.8]. Protein extraction of both the cytosolic and mitochondrial fractions was performed using a multiple centrifugation method as described previously (31,32).

Medium from treated cells was harvested, spun at 800 x g for 5 min and supernatant filtered (0.45 μ m). Membrane fraction lysates were prepared as described previously. Proteins were precipitated with trichloroacetic acid. Protein samples were denatured by boiling for 5 min with an equal volume of 2X Tris-glycine SDS buffer. Protein was separated by 7-12% SDS-PAGE and transferred to nitrocellulose membrane (Millipore). After blocking with 5% non-fat milk/PBS-T for 3 h at room temperature, the membranes were incubated with the primary antibodies overnight at 4°C. Then, fluorescently labeled secondary antibody (Rockland, Limerick, PA, USA) was added for 1 h and subsequently scanned by the Odyssey Infrared Imaging system (LI-COR Biosciences, Waltham, MA, USA). In these experiments, β -actin and COX IV were used as loading controls for the whole cellular and mitochondrial proteins, respectively.

Immunofluorescence. Cardiomyocytes were grown in 48-well plates. After respective treatments for 4 h, cardiomyocytes were washed twice with PBS, and fixed with freshly prepared 4% paraformaldehyde at room temperature for 15 min. Antigen accessibility was increased by treatment with 2% Triton X-100 for 10 min. Then cardiomyocytes were blocked with 3% BSA for 30 min. Following incubation with primary and secondary antibodies, cardiomyocytes were incubated with primary antibodies overnight at 4°C. After washing, cardiomyocytes were stained with a secondary antibody for 1 h at room temperature. Each time after the operation, cardiomyocytes were washed thrice with PBS, and each time for 5 min. Cardiomyocytes were captured and analyzed using TCS SP8 STED 3X (Leica, Wetzlar, Germany).

Analysis of PA using gas chromatography-mass spectrometry (GC-MS). GC-MS analysis was performed on an Agilent 7890A gas chromatograph coupled with an Agilent 5975C Series MSD (Agilent Technologies, Palo Alto, CA, USA). An Agilent DB-23 column (60 m x 0.25 mm x 0.15 μ m) was used for separation. The initial oven temperature was 50°C for 1 min and then raised to 178°C at 8°C/min for 4 min, followed by further increases at 4°C/min to 186°C, 1°C/min to 190°C for 1 min and 15°C/min to 220°C for 10 min. The injection column was 1 μ l in splitless mode. The helium carrier gas flow rate was set at 1 ml/min. Detector voltage of EI was 70eV and the mass range was set at m/z 50-550.

Transmission electron microscopy (TEM). Samples of heart tissue were fixed with 2.5% glutaraldehyde overnight. Subsequently, samples were incubated while protected from light 1% osmium tetroxide for 2 h. After washing in distilled water, the samples were incubated in 2% uranyl acetate for 2 h at room temperature and then dehydrated in grades ethanol concentrations. Finally, the samples were embedded in molds with fresh resin. Ultrathin sections were obtained with an EM UC7 (Leica), stained with lead citrate and examined with a Tecnai G2 T12 (FEI, USA).

ELISA analysis. After respective treatments, cardiomyocytes were collected and, after addition of phenylmethylsulfonyl fluoride (PMSF), then the medium from treated cardiomyocytes was harvested, spun at 800 x g for 5 min and supernatant

filtered (0.45 μ m). Samples were transferred to antibody-coated plates. The concentration of DAG was determined by competitive inhibition ELISA. Plate preparation and assay procedure were performed according to the manufacturer's recommendations. The absorbance was read with a reference wavelength of 450 nm. DAG concentration for each sample was calculated after generating a standard curve by a microplate reader (BioTek Synergy2; BioTek, Winooski, VT, USA).

Cardiomyocyte viability tests. Cardiomyocytes were seeded in 96-well plates at a concentration of 1×10^4 cells/ml. After treatment, cell viability was tested by using the CCK-8 kit. Absorbance at 450 nm was measured with a microplate reader (BioTek Synergy2; BioTek).

LDH release assay. The cardiomyocytes were seeded in 96-well plates (1×10^4 cells/ml) and treated. The activity of LDH in cardiomyocytes released into the medium following treatment was assessed as previously described by a microplate reader (BioTek Synergy2; BioTek) analysis at 440 nm using an LDH cytotoxicity assay kit, according to the manufacturer's instructions.

Three-dimensional surface reconstruction and mitochondrial volume calculations. Three-dimensional surface-reconstruction image raw data sets were collected by spinning-disc confocal microscopy. The Z-Stack was deconvolved and three-dimensional surface reconstruction was carried out with IMARIS 7.0.0 software (Bitplane, South Windsor, CT, USA). All confocal microscopy was carried out on a confocal laser scanning microscope (Olympus FV10i; Olympus, Tokyo, Japan). Confocal slice thickness was typically kept at 0.6 μ m consistently for each fluorescence channel, with ten slices typically being taken to encompass the three-dimensional entirety of the cells in the field of view. Maximum-intensity projections of each region were calculated for subsequent quantification and analysis. To quantify mitochondrial volume, images were deconvolved with ImageJ and Z-Stack analysis of the threshold images volume-reconstituted using the VolumeJ plug-in, and volumes of mitochondria were quantified using the ImageJ-3D object counter plug-in. Care was taken to ensure consistency of thresholding over multiple fields of view and samples. Once this process was complete, Object Counter 3D under the particle analysis algorithm within ImageJ was employed to measure the volume of mitochondria and area of cells within a specified region of interest. Calculation for the adjusted total mitochondrial volume per cell was as follows: (percentage of total volume of mitochondria)/(percentage of total area of cell).

Statistical analysis. The results are expressed as the mean \pm standard deviation (SD). Statistical analysis of the results was carried out using the repeated-measures analysis of the variance (ANOVA) or two-way ANOVA followed by the Tukey's Honestly Significant Difference (HSD) test for multiple comparisons. The significance level was set at $p < 0.05$.

Results

PEDF decreases the mitochondrial density of primary cardiomyocytes under hypoxic condition. We have previously

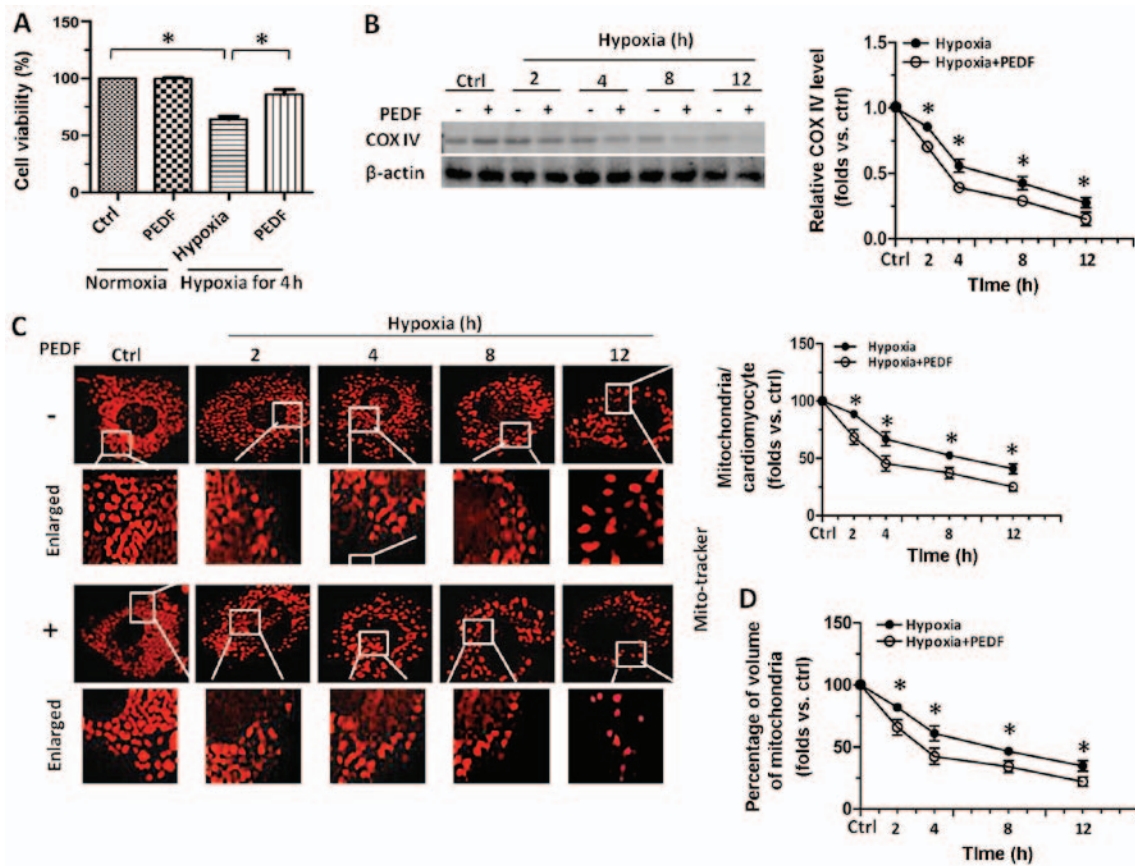


Figure 1. Pigment epithelial-derived factor (PEDF) decreased the mitochondrial density of primary cardiomyocytes under hypoxic condition. Primary cardiomyocytes were maintained in normoxic or hypoxic conditions for 0, 2, 4, 8 and 12 h with or without PEDF (10 nM). (A) Cell viability was assayed by the cell counting kit-8 (CCK-8). Approximately 1×10^4 cells were grown in each well of 96-well plates with 100 μ l medium and the absorbance at 450 nm was directly proportional to the number of viable cells ($n=4$, $*p<0.05$). (B) Samples were collected for western blotting to analyze the level of mitochondrial protein COX IV by ImageJ software ($n=4$, $*p<0.05$). (C) Samples were stained with MitoTracker Red. For each indicated times, total mitochondrial number per cultured cardiomyocytes was quantified from stacks of images through the entire thickness of cardiomyocytes by ImageJ software ($*p<0.05$, $n=90$ cardiomyocytes from three independent experiments; scale bar, 20 μ m). (D) Five randomly picked regions of each sample were captured by confocal z-axis scanning and the total volume of mitochondria was calculated and quantified ($*p<0.05$, $n=90$ cardiomyocytes from three independent experiments; scale bar, 20 μ m). Data are expressed in fold induction, relative to control. Values are means \pm SD.

documented that PEDF has a protective effect against hypoxia-induced apoptosis and necroptosis in primary cardiomyocytes and H9c2 cells (7,33). To further confirm the observation that PEDF exerts a protective effect in hypoxic primary cardiomyocytes, CCK-8 assays were performed. As shown in Fig. 1A, onset of hypoxia up to 12 h led to an immediate decrease in the viability of cardiomyocytes, while treatment with PEDF resulted in a marked increase in the viability of cardiomyocytes compared with hypoxia group. Compared with hypoxia group at the indicated times, cardiomyocytes treated with PEDF resulted in a marked decrease in the level of COX IV (Fig. 1B), which is used as an index of mitochondrial density. Besides, mitochondrial density analyzed by immunostaining with MitoTracker Red in PEDF-treated cardiomyocytes was much less than that in control groups (Fig. 1C). We also quantitatively measured the total mitochondrial volume within the cardiomyocytes by a commonly used three-dimensional imaging technique followed by analysis with ImageJ software. As expected, the total mitochondrial volume was significantly reduced after addition of PEDF compared with hypoxia group (Fig. 1D). Taken together, the effect of PEDF on decreasing mitochondrial density of hypoxic cardiomyocytes were first observed and the results here suggest that compared

with hypoxia group, PEDF could further reduce the mitochondrial density of primary cardiomyocytes.

PEDF plays a protective role in primary cardiomyocytes through promoting mitophagy, and had no effect on mitochondrial fusion. Mitophagy and mitochondrial fusion are closely related with the change of mitochondrial number (8,9,34,35). Therefore, we investigated the role of mitophagy and mitochondrial fusion in PEDF-decreased mitochondrial density of hypoxic cardiomyocytes. As shown in Fig. 2A, with the addition of PEDF, we observed an increase of LC3-II level at the various time points. PEDF groups reached a maximum after 4 h hypoxia, while hypoxia groups reached a maximum after 8 h. However, the levels of Opa1, Mfn1 and Mfn2 had no significant difference between the two groups throughout the observation periods. Besides, we found that PEDF could induce the increase of mitophagic flux (Fig. 2B). The results of CCK-8 assays showed that treatment with PEDF up to 4 h resulted in a marked increase in the viability of cardiomyocytes compared with hypoxia group, while the addition of lysosome inhibitor BAF1 (24,26,36) significantly reduced the viability of cardiomyocytes compared with PEDF-treated cardiomyocytes (Fig. 2C). LDH released assays obtained a

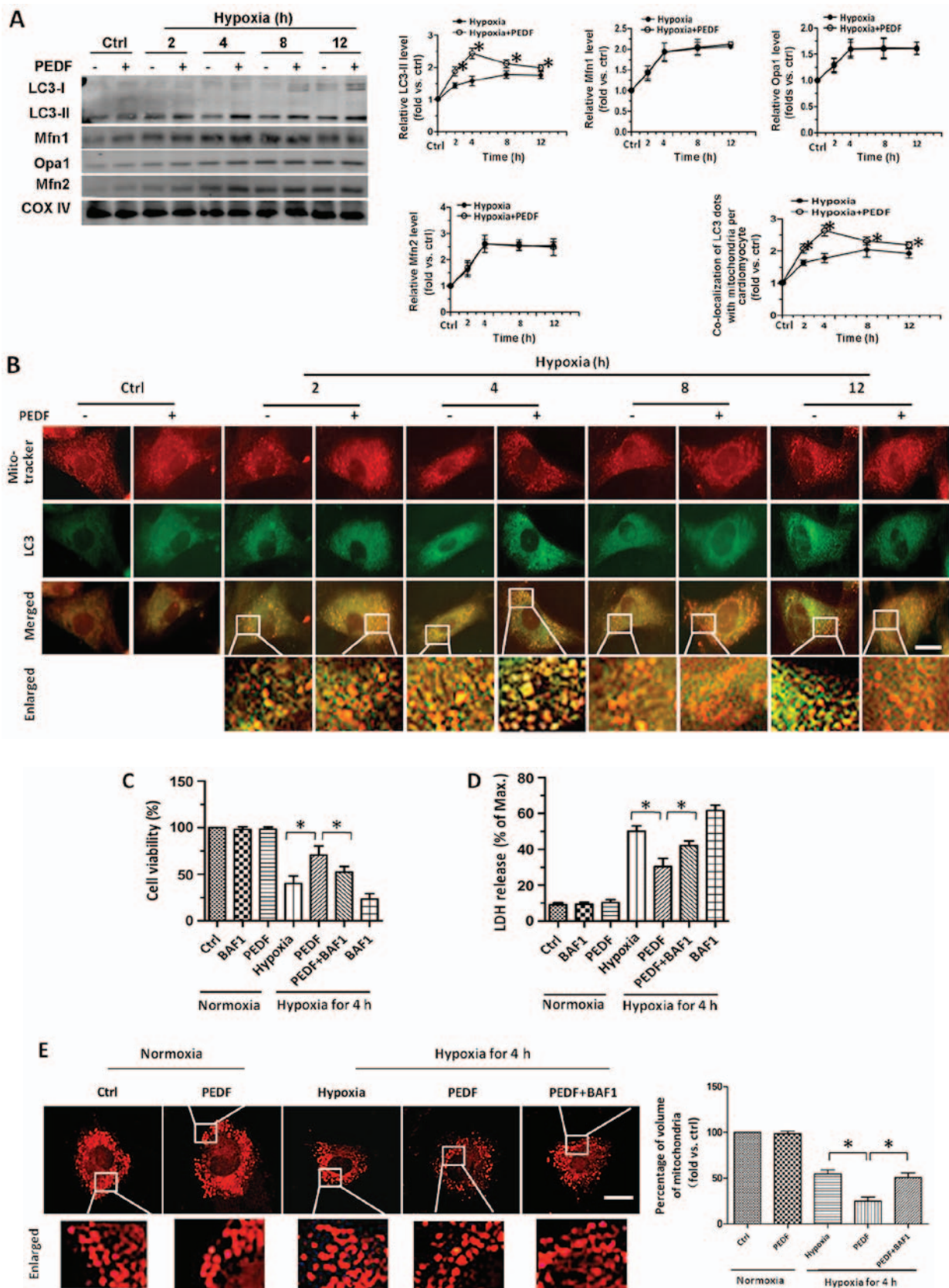


Figure 2. Pigment epithelial-derived factor (PEDF) played a protective role in primary cardiomyocytes through promoting mitophagy, and has no effect on mitochondrial fusion. Primary cardiomyocytes were maintained in normoxic or hypoxic conditions for 0, 2, 4, 8 and 12 h with or without PEDF (10 nM). Lysosome inhibitor BAF1 (50 nM) was added. (A) LC3-II, Opa1, Mfn1 and Mfn2 proteins in the mitochondrial fractions were tested by western blotting (n=4, *p<0.05). (B) Samples were stained with MitoTracker Red and anti-LC3 (green) antibody. Quantitative analysis of cardiomyocytes that contain co-localization of LC3 dots with mitochondria per cardiomyocytes. (*p<0.05, n=90 cardiomyocytes from three independent experiments; scale bar, 20 μ m). Lysosome inhibitor BAF1 (50 nM) was added. (C and D) Cell counting kit-8 (CCK-8) and LDH released assays were employed to assessed cell viability and the rate of cell death (n=4, *p<0.05). (E) Samples were stained with MitoTracker Red. Five randomly picked regions of each sample were captured by confocal z-axis scanning and the total volume of mitochondria was calculated and quantified (*p<0.05, n=90 cardiomyocytes from three independent experiments; scale bar, 20 μ m). Data were expressed in fold induction, relative to control. Values are means \pm SD.

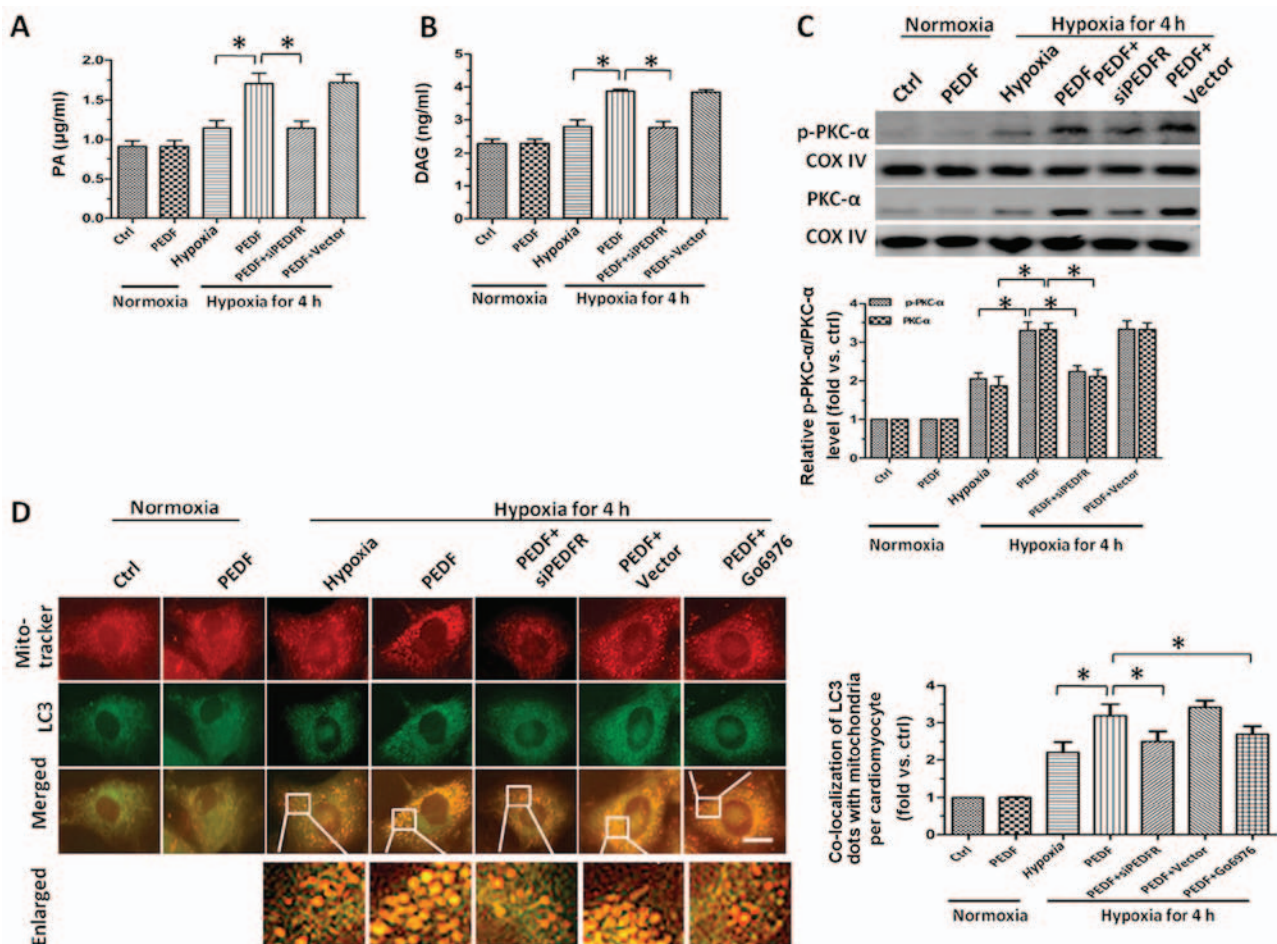


Figure 3. Pigment epithelial-derived factor (PEDF) increases the level of PA and diacylglycerol (DAG) via PEDF-R, leading to protein kinase C α (PKC- α) activation, which induced mitophagy. Primary cardiomyocytes were maintained in normoxic or hypoxic conditions for 4 h with or without PEDF (10 nM). RNA interference assays were used to silence PEDF-R. (A) The PA level was quantified using GC-MS ($n=4$, * $p<0.05$). (B) The DAG level was quantified by enzyme-linked immunosorbent assay (ELISA) ($n=4$, * $p<0.05$). (C) p-PKC- α and PKC- α proteins were analyzed by western blotting ($n=4$, * $p<0.05$). (D) PKC- α inhibitor Go6976 (1 μ M) was added. Samples were stained with MitoTracker Red and anti-LC3 (green) antibody. Quantitative analysis of cardiomyocytes that contain co-localization of LC3 dots with mitochondria per cardiomyocytes (* $p<0.05$, $n=90$ cardiomyocytes from three independent experiments; scale bar, 20 μ m). Data are expressed in fold induction, relative to control. Values are means \pm SD.

similar conclusion (Fig. 2D). Fig. 2E shows the total mitochondrial volume was markedly decreased after addition of PEDF compared with hypoxia group, while the addition of BAF1 significantly increased the mitochondrial volume of cardiomyocytes. The results demonstrated that PEDF can promote hypoxic cardiomyocyte mitophagy, and has no significant effect on mitochondrial fusion. More importantly, these results also suggest the possibility that PEDF-induced mitophagy is important for cell survival under hypoxic condition.

PEDF increases the level of PA and DAG via PEDF-R, leading to PKC- α activation, which induced mitophagy. Since we had confirmed PEDF could reduce the mitochondrial density of cardiomyocytes through promoting mitophagy, we attempted to identify the pathway underlying PEDF-induced hypoxic cardiomyocytes mitophagy. An earlier study showed that PEDF could stimulate cardiac lipid degradation via PEDF-R (15). Therefore, we next investigated the lipolysis of PEDF in hypoxic cardiomyocytes. After 4 h hypoxia, we found the level of PA increased significantly after treated with PEDF compared with hypoxia group (Fig. 3A). Once taken by cells, excess FFAs can be converted into their respective acyl-

CoA derivatives and then incorporated and stored in the cells as neutral lipids like DAG (37-39). Therefore, we measured the level of DAG in cardiomyocytes and found that treatment with PEDF led to a marked increase of DAG (Fig. 3B). As one of the well-established pathway activated by DAG is the PKC family (40), we next evaluate the expression and activation of PKC- α . As shown in Fig. 3C, we found PEDF not only upregulated expression of PKC- α , but also increased its activation. After 4 h hypoxia, Fig. 3D showed the number of LC3 and MitoTracker puncta in PEDF-treated cardiomyocytes was significantly more than that in control group. With the addition of PKC- α inhibitor Go6976, the puncta were decreased. Taken together, such findings demonstrated that the upregulation and activation of PKC- α resulted from the increase of PA and accumulation of DAG is implicated in PEDF-induced mitophagy and the activation of PKC- α is involved as upstream signal for the promotion of mitophagy in primary cardiomyocytes stimulated with PEDF under hypoxic condition.

PEDF-activated PKC- α promotes FUNDC1-mediated primary cardiomyocyte mitophagy under hypoxic condition. After

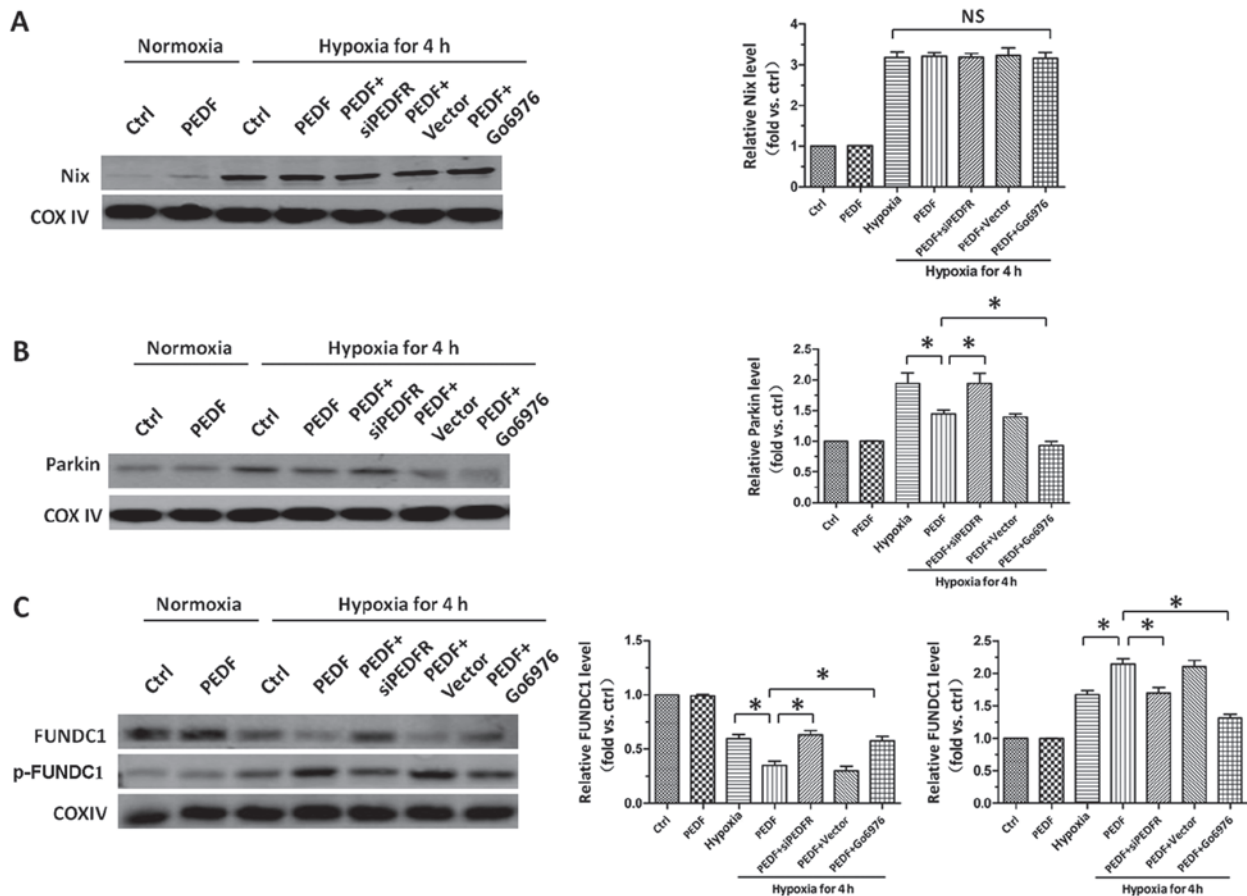


Figure 4. Pigment epithelial-derived factor (PEDF)-activated protein kinase α (PKC- α) promotes FUNDC1-mediated primary cardiomyocyte mitophagy under hypoxic condition. Primary cardiomyocytes were maintained in normoxic or hypoxic conditions for 4 h with or without PEDF (10 nM). RNA interference assays were used to silence PEDF-R. PKC- α inhibitor Go6976 (1 μ M) was added. (A-C) Nix, Parkin, FUNDC1 and p-FUNDC1 proteins in the mitochondrial fractions were tested by western blotting (n=4, *p<0.05). Data were expressed in fold induction, relative to control. Values are means \pm SD.

establishing the role of PKC- α in PEDF-induced hypoxic cardiomyocytes mitophagy, we then tried to investigate the downstream molecular mechanism underlying PKC- α -regulated mitophagy. As shown in Fig. 4A, we found hypoxia increased the level of Nix, but compared with hypoxia group, PEDF failed to increase the level of Nix. It has been established that decreased recruitment of Parkin to mitochondria suppress mitophagy. The result showed PEDF downregulated the level of Parkin, suggesting PEDF could inhibit hypoxia-induced Parkin mitochondrial translocation, which indicated PEDF suppress Parkin-mediated mitophagy (Fig. 4B). After precluding the effects of Parkin and Nix, we then examined the effect of PEDF on FUNDC1 and p-FUNDC1. We found that PEDF decreased the level of FUNDC1 and increased the level of p-FUNDC1, while Go6976 (18) was capable of effectively increasing the level of FUNDC1 and decreasing the level of p-FUNDC1 (Fig. 4C). Collectively, these data indicate that PEDF is able to increase the level of p-FUNDC1 via activation of PKC- α and then induce mitophagy, rather than through Parkin and Nix pathways.

PEDF-induced hypoxic primary cardiomyocyte mitophagy through FUNDC1 is dependent on ULK1 pathway. The ULK1 signaling pathway has been well established as the crucial regulator of FUNDC1-induced mitophagy (26). Therefore, we next examined the role of ULK1 signaling pathway in PEDF-induced mitophagy. Our data demonstrated that PEDF

could markedly increase the level of ULK1, and significantly increase the level of p-FUNDC1 in hypoxic cardiomyocytes. Besides, we also observed inhibition of PKC- α by inhibitor Go6976 capable of effectively suppressing the increase of ULK1, and inhibiting the increase of p-FUNDC1 (Fig. 5). These results showed that FUNDC1-induced mitophagy which mediated by PEDF depends on the ULK1 signaling pathway. PEDF decreased the mitochondrial density by promoting mitophagy *in vivo*. After concluding PEDF could decrease the mitochondrial density of cardiomyocytes via promoting mitophagy *in vitro*, we tested whether PEDF could promote cardiomyocytes mitophagy *in vivo*. Transmission Electron Microscopic observation in rats treated with PEDF showed a marked increase in the number of mitophagy compared with control group (Fig. 6A). We also found that in border zones the number of mitochondria and volume of mitochondria in PEDF-treated rats was less than that in the control group (Fig. 6B). These findings indicate that under ischemic condition, PEDF could promote mitophagy and decrease the mitochondrial density in *in vivo* model of AMI.

Discussion

Both in vitro and in vivo studies, we first found PEDF could promote mitophagy and decrease the mitochondrial density of hypoxic primary cardiomyocytes. Importantly, PEDF protects

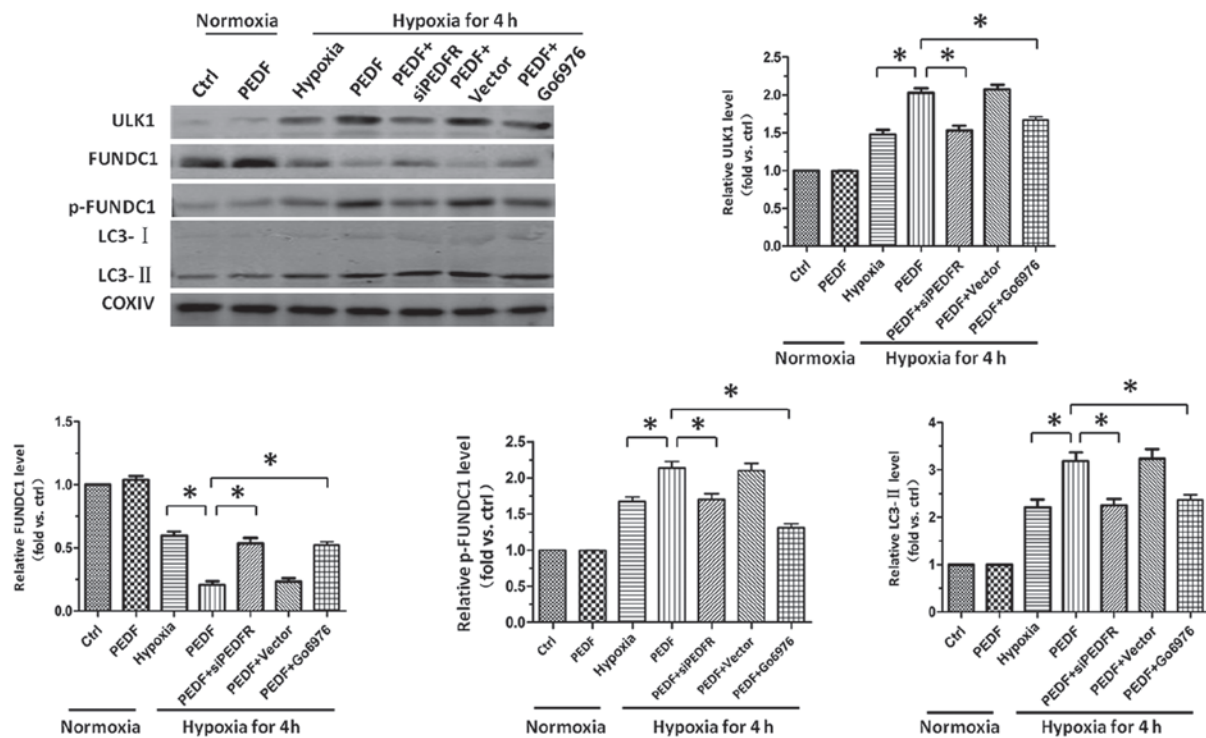


Figure 5. Pigment epithelial-derived factor (PEDF)-induced hypoxic primary cardiomyocyte mitophagy through FUNDC1 is dependent on ULK1 signaling pathway. Primary cardiomyocytes were maintained in normoxic or hypoxic conditions for 4 h with or without PEDF (10 nM). RNA interference assays were used to silence PEDF-R. PKC- α inhibitor Go6976 (1 μ M) was added. ULK1, FUNDC1, p-FUNDC1 and LC3-II levels were tested by western blotting (n=4, *p<0.05). Data were expressed in fold induction, relative to control. Values are means \pm SD.

cardiomyocytes through promoting hypoxic cardiomyocyte mitophagy. We identified a novel signaling pathway, PEDF/PEDF-R/PA/DAG/PKC- α /ULK1/FUNDC1, in regulating PEDF-promoted mitophagy. Based on the findings from this study, in conjunction with those from previous studies (7,33), it is conceivable that PEDF may represent a promising therapeutic approach for ischemic heart disease.

Accumulating evidence has shown that hypoxia can induce a loss of mitochondrial density (41-43). Mitophagy and mitochondrial fusion are closely related with the change of mitochondrial number (8,9,34,35). The number of mitochondria within a cell are controlled by precisely regulated rates of organelle fusion (35) and mitophagy is a process through which damaged mitochondria are selectively eliminated by autophagy (44). As this study shows, hypoxia did reduce the total mitochondrial volume and increase the level of fusion protein. However, there is no significant difference between PEDF group and hypoxia group. So we speculate that PEDF has no effect on mitochondrial fusion, and PEDF decrease the mitochondrial density of hypoxic cardiomyocytes through promoting mitophagy, rather than mitochondrial fusion.

In the endogenous protective effects of hypoxic cells, mitophagy could attenuate the damage of ROS and cytochrome *c* by eliminating the injured mitochondria (45). Moreover, mitophagy also could reduce ATP consumption and maintain metabolic stability (46). In this study PEDF promotes and enhances hypoxic cardiomyocyte mitophagy, which means PEDF could eliminate the damaged mitochondria earlier and greatly via promoting hypoxic cardiomyocyte mitophagy. Besides, considering the role of mitochondria in cell ATP generation, we propose that PEDF-promoted

mitophagy may be a potentially protective mechanism and play an earlier protective role in cardiomyocytes. Results from this study showed that PEDF was able to decrease the mitochondrial density of hypoxic cardiomyocytes earlier and greatly, compared with hypoxia only, which means PEDF could alleviate the damage caused by injured mitochondria. Therefore, it is significant that PEDF reduces the mitochondrial density of hypoxic cardiomyocytes, and plays a crucial role in cardiomyocyte protection.

In this study we found PEDF could promote the level of PA. As is known, PA is a saturated fatty acid known to cause lipotoxicity in cells (18), and several factors such as ROS production (47) have been implicated in lipotoxicity. However, in this study PA promotes hypoxic cardiomyocyte mitophagy without lipotoxicity. We previously reported that PEDF could increase the level of FFAs in cardiomyocytes after AMI via binding to PEDF-R (15), a lipase-linked cell membrane receptor for PEDF (14). When taken by cells, excess FFAs can be converted into their respective acyl-CoA derivatives (38) which can be incorporated and stored in the cells similarly to DAG (37,39). Consistent with this, we found the increase of PA caused the accumulation of DAG.

Previous study noted that DAG could serve as a natural agonist to recruit PKC proteins to membrane for activation (40). The PKC family and/or lipid-activated serine-threonine protein kinase function downstream of many signal transduction pathway (19,20). PKC- α is the predominant conventional PKC isoform expressed in the mouse, rat, and human heart (21,22). Our findings showed DAG not only upregulated PKC- α , but also induced its activation, which is inconsistent with earlier study that DAG can only induce the activation of PKC- α in

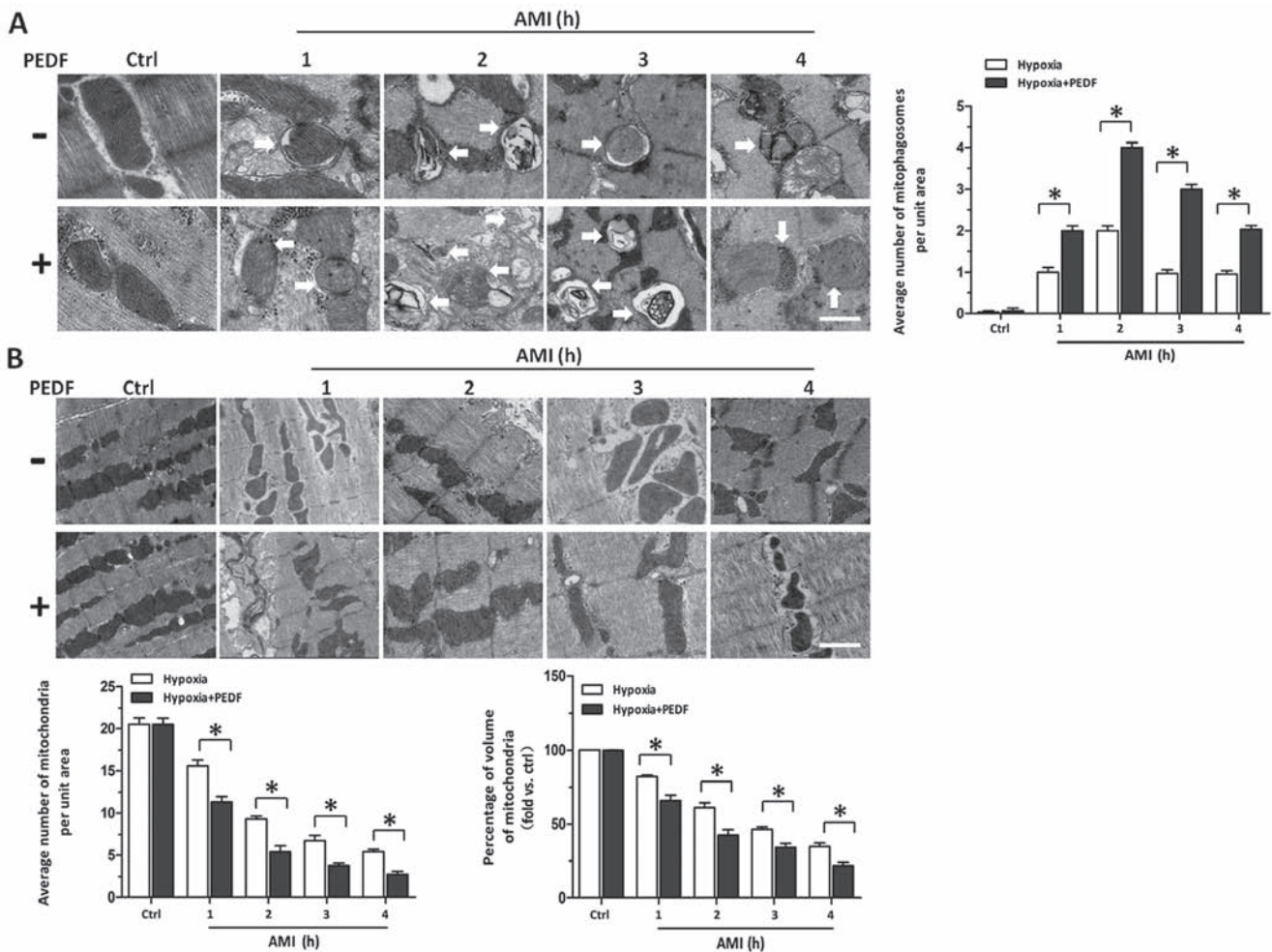


Figure 6. Pigment epithelial-derived factor (PEDF) decreases the mitochondrial density by promoting mitophagy *in vivo*. Among the 55 rats entered into our experiment, 5 died after 4 h surgery. So 50 rats were used for analysis of transmission electron microscopy and randomized into the four group (n=5). (A) Electron micrographs show cardiomyocyte mitophagy from border zone after 1, 2, 3 and 4 h surgery. Typical mitophagy (arrows) was observed. Scale bar, 500 nm. (B) Electron micrographs show cardiomyocyte mitochondrial density and mitochondrial volume from border zone after 1, 2, 3 and 4 h surgery. Scale bar, 2 μ m.

mouse embryonic fibroblasts and HepG2 cells (18). We postulate that the conflicting results may be attributed to factors such as the cell types used, the concentration and time of PA induced by PEDF. Taken together, it is believed that PEDF can promote mitophagy via activation of PKC- α dependent increase of PA and accumulation of DAG.

In this study we also found that the activation of PKC- α was associated with mitophagy. Mitophagy is mainly mediated by three signaling pathways, namely, Parkin, Nix and FUNDC1 (23-25). Early studies have suggested that endogenously or ectopically Parkin could be recruited towards the depolarized mitochondria through interaction with PINK1 (23,48). As mitochondrial membrane potential ($\Delta\psi$) is lost, PINK1 is stabilized and accumulates on the outer membrane of damaged mitochondria where it selectively recruits Parkin (48). FUNDC1, an outer mitochondrial membrane protein, promotes mitophagy through interaction with LC3 in response to hypoxia (24). Nix is also a hypoxia-responsive protein and acts as a mitophagy receptor which is necessary for erythroid maturation through autophagic removal of unwanted mitochondria (49). Data from this study showed PEDF has no effect on Nix, and PEDF cannot enhance mitophagy through Parkin, because PEDF could inhibit the decline of mitochondrial membrane potential (33). The

results revealed PEDF could increase the level of p-FUNDC1 compared with hypoxia group, and then promote mitophagy. So it is believed that PEDF could promote mitophagy through FUNDC1 via activating PKC- α . It is beneficial to further investigate mitophagy. Recent studies have suggested that damaged and unwanted mitochondria can be removed by autophagy (23). Therefore, based on the findings from this study, in conjunction with those from previous studies, it is conceivable that PEDF-promoted mitophagy through FUNDC1 is essential for mitochondrial quality control in cardiomyocytes under hypoxic condition. Besides, although recent studies suggest SRC and ULK1 are critical for the FUNDC1-mediated mitophagy, we found that PEDF induced mitophagy is dependent on ULK1 pathway, rather than SRC pathway.

Taken together and to the best of our knowledge, data presented in this study clearly demonstrate for the first time that PEDF-promoted mitophagy constitutes an underlying protective response against hypoxia in primary cardiomyocytes. However, future studies are needed to fully understand whether PEDF-promoted mitophagy represents a means to reduce ischemic injury *in vivo*. Besides, this study did not pursue the search for molecular mechanism in which PKC- α modulates the expression levels of ULK1. Therefore, future

investigation is needed to clarify these detailed mechanisms. Because mitophagy has been linked to cardioprotective benefit, data from this study also provide insights into a promising future therapeutic strategy in cardiac pathologies.

Acknowledgements

We would like to express our sincere gratitude to Dr Xiaofang Yang (Laboratory of Clinical and Experimental Pathology, Xuzhou Medical University) for providing the anti-p-FUNDC1 (Ser-17) polyclonal antibody which was used for detecting phosphorylated FUNDC1.

Funding

This study was supported by the National Nature Science Foundation of China (nos. 81570242 and 81270173) and the Technology Bureau of Xuzhou of China (grant no. KC14SH106).

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All the authors conceived and designed the study protocol. YL, ZL and YZ performed experiments. YL, ZL, YZ and XW analyzed the data. YL, ZL, YZ, XW and PL interpreted the results of the experiments. YL prepared the figures. YL, ZL, YZ, XW, PL, QZ, HZ, ZW, HD and ZZ approved the final version of manuscript. HD and ZZ conceived and designed the research. HD and ZZ edited and revised manuscript. ZZ drafted the manuscript. All the authors gave final read and approved the final manuscript.

Ethics approval and consent to participate

All the procedures were performed following the guidelines of the Directive 2010/63/EU of the European Parliament, and have been approved by the Ethics Committee for Animal Experimentation of the Institutions where experiments were carried out.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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