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Original Article

Pigment epithelium derived factor (PEDF) prevents methyl methacrylate monomer-induced cytotoxicity in H9c2 cells

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Abstract

Acrylic bone cements are currently the most frequently and extensively used materials in orthopedic implant treatment. However, adverse effects have been described of acrylic bone cement on the cardiovascular system. In the present study, we examined the cytotoxicity of bone cement ingredient methyl methacrylate (MMA) to cardiomyocytes and the potential detoxifying effect of pigment epithelium-derived factor (PEDF) in H9c2 cells. We found that high concentration of MMA ($>$ 120 mmol/L) led to necrotic cell death in H9c2 cells. However, MMA at low concentrations (30-90 mmol/L) caused apoptosis. Pretreatment of PEDF prevented MMA-induced cytotoxicity. In addition, PEDF enhanced total superoxide dismutase activities, and decreased MMA-induced production of malonaldehyde. Furthermore, MMA-induced downregulation of Akt activity was suppressed by PEDF. PEDF also increased the levels of peroxisome proliferator activated receptor gamma (PPARγ) and lysophosphatidic acids (LPA) through PEDF receptor. These results indicated that PEDF inhibited MMA-induced cytotoxicity through attenuating oxidative stress, activating the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and/or PEDF receptor-LPA-PPARγ pathways in H9c2 cells. PEDF may be explored as a candidate therapeutic agent for alleviating bone cement implantation syndrome during orthopedic surgery.

Keywords: pigment epithelium-derived factor, oxidative stress, bone cement, methyl methacrylate

Introduction

Bone cement implantation syndrome (BCIS) is a rare but potentially lethal intra-operative complication during cemented orthopedic surgeries[1]. It is characterized by hypoxia[2], hypotension[3–4], cardiac arrhythmias[5], increased pulmonary vascular resistance and cardiac arrest[6–7], resulting in immediate death in 0.1% of the

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recipients. Although BCIS has been reported for 46 years, the etiology and pathophysiology of BCIS are still not fully clear. Several mechanisms have been proposed, one of which is the release of acrylic resin-based bone cement materials into the circulation following cementation.

Acrylic resin-based bone cements primarily consist of a solid part of a prepolymerized polymethyl methacrylate (PMMA) and a liquid part of methyl methacrylate (MMA). Various studies have shown adverse effects of acrylic bone cement on body. There are potential neurotoxic effects of PMMA during cranioplasty[8–9]. Bone cement could induce apoptosis and secondary necrosis in osteoblastic cell lines[10–11]. Moreover, oxidative stress is a causal factor for cell damage after exposure to bone cement or bone cement ingredients. *N*-Acetyl cysteine, a well-known antioxidant, could suppress PMMA-induced cell death in rat primary osteoblasts[12]. It is also reported that MMA reduces myocardial contractility in isolated dog heart muscle model[13]. However, the adverse effects of acrylic bone cement at the cellular level in cardiomyocytes are still unknown.

Pigment epithelium-derived factor (PEDF) is a secreted glycoprotein that belongs to the non-inhibitory serpin family group[14]. A body of evidence indicates that PEDF exerts diverse physiologic activities, such as anti-angiogenic, anti-tumorigenic, anti-inflammatory, antioxidant, neurotrophic and neuroprotective properties. PEDF could inhibit hyperglycemia-induced apoptotic cell death via activating phosphatidylinositol 3-kinase (PI3K)/Akt pathway in goat retinal pericytes[15]. In addition, PEDF was able to attenuate hyperglycemia-induced reactive oxygen species (ROS) in pericytes[16].

Although the antioxidant capacity of PEDF has been well studied in many other fields, there are few studies on the potential protective effects of the agent on bone cement extracts-induced cytotoxicity. Thus, the present study was designed to test the cytotoxicity of MMA to cardiomyocytes and the potential protective effects of PEDF on cardiomyocytes.

Materials and methods

Reagents

Anti-p-Akt antibody (#0458), anti-cleaved caspase-3 (#9664), and anti-β-actin (#13E5) antibodies were purchased from Cell Signaling Technology, USA. PPARg (#ab70405) was purchased from Abcam, Inc. (Abcam, Cambridge,UK). Annexin V-FITC/PI Staining Kit was purchased from Roche, Switzerland. Atglistatin (#SML1075), methyl methacrylate monomer (#55909), necrostatin-1 (#N9037), Hoechst33342 (#861405) and 2`,7`-dichlorofluorescin diacetate (DCFH-DA;#D6883) were purchased from Sigma, USA. Superoxide dismutase (SOD; #A001-1) and malonaldehyde (MDA; #A002-1) detection kit were purchased from JIANCHENG Bioengineering Institute, China. Recombinant rat PEDF was synthesized by CUSABIO BIOTECH CO. Ltd, China. Cell Counting Kit (CCK-8) was from Dojindo Molecular Technologies (Kumamoto, Japan).

Cell culture, PEDF preincubation and MMA treatment procedure

The embryonic rat heart derived H9c2 cells was obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and cryopreserved at School of Medicine, Jiangsu University (Zhenjiang, Jiangsu, China). The cells were cultured in DMEM (Hyclone, Thermo, USA) supplemented with 10% fetal bovine serum (Hyclone, Thermo, USA), 50 U/mL penicillin and 50 mg/mL streptomycin at 37°C at an atmosphere of 95% air and 5% CO2. The cells were trypsinized, plated in 96 well plates or cell culture disks for 24 hours, preincubated with or without PEDF(10 nmol/L) for half an hour, and then began to contact with MMA in serum-free DMEM. Then, the cells were collected and washed two times with PBS and then tested by CCK-8 assay and Annexin V-FITC/propidium iodide (PI) staining.

CCK-8 cell viability assay

H9c2 cells were seeded in 96-well plates at a concentration of $1x10^4$ cells/mL. After treatment, cell viability was detected by using the CCK-8 kit. Absorbance at 450 nm was measured with a microplate reader (BioTek Synergy2, VT). The means of the optical density (OD) measurements from 6 wells of the indicated groups were used to calculate the percentage of cell viability.

Annexin V-FITC/propidium iodide staining

H9c2 cells were cultured in 24-well plates. After treatment, the cells were washed three times with PBS gently, then 500 mL of cold binding buffer, 5 mL of Annexin V-FITC and 5 mL of propidium iodide (PI) were added to the wells at room temperature in the dark. Nuclei were stained with Hoechst 33342. The sample was observed by a fluorescence microscope system (Olympus, Japan).

Western blotting analysis

For Western blotting analysis, the cells were solubilized in lysis buffer (100 mmol/L Tris-HCl, 4% SDS, 20% glycerine, 200 mmol/L DTT and protease

inhibitors, pH 6.8). The protein concentrations in the supernatants were measured by BCA protein assay. Protein was separated by 10%, 12% or 15% SDS-PAGE and transferred to nitrocellulose membrane (Millipore, USA). After blocking with 5% non-fat milk/PBS-T for 3 hours at room temperature, the membranes were incubated with primary antibodies (dilution of 1:100). Then, fluorescently labeled secondary antibody (Rockland, USA) was added for 1 hour and subsequently scanned by the Odyssey Infrared Imaging System (Li-Cor Biosciences, USA).

Detection of intracellular ROS production

Intracellular ROS was measured by 2`7`-dichlorofluorescein (DCF). 2`7`-dichlorodihydorofluorescein diacetate (DCFH-DA; Sigma, St. Louis, MO) is cell-permeant. It can enter the cell where intracellular esterases cleave off the diacetate group. The resulting DCFH is retained in the cytoplasm and oxidized to DCF by ROS. After contact with MMA, cells were then washed once with phenol red-free medium, and incubated in 200 mL working solution of DCFH-DA (20 mmol/L) at 37°C for 30 minutes. The cells were observed under a fluorescence microscope (Olympus, Japan). The fluorescence of DCF was monitored at the excitation and emission wavelengths of 485 nm and 530 nm.

Measurement of total superoxide dismutase (T-SOD) activities and methane dicarboxylic aldehyde (MDA) levels

T-SOD activities and MDA levels were measured using respective detection kits according to the manufacturers' instructions. Data were observed using multi-mode microplate reader (Synergy 2, Bio-Tek, USA).

Detection of lysophosphatidic acids (LPA) concentration by ELISA

After treatment with MMA, the cell culture medium was collected and, after addition of phenylmethlsulfonyl fluoride (PMSF; 1 mmol/L) as a serine protease inhibitor, was precleared by centrifugation (25 minutes; 13,000 rpm). Samples were transferred to LPA monoclonal antibody-coated plates. The concentration of LPA was determined by sandwich ELISA kit (CEK623Ge, Cloud-Clone Corp, Wuhan China). Plate preparation and assay procedure were performed according to the manufacturer's recommendations using tetramethylbenzidine as a substrate. The absorbance at 455 nm was read with a reference wavelength of 650 nm using an ELISA plate reader.

Data analysis

Graphical presentation and statistical analysis were made by GraphPad prism version 5.0 software (GraphPad Inc., San Diego CA). Values are expressed as mean±S.E.M. of at least 3 independent experiments. Statistical analysis of the results was carried out using the Student's *t*-test or one-way analysis of the variance (ANOVA). Statistical significance was determined at $P < 0.05$.

Results

PEDF attenuated MMA-induced cell death in H9c2 cells

To determine the acute effect of MMA on cell survival rate, H9c2 cells were subjected to increasing concentrations of MMA for 2 hours and examined by CCK-8 assay. As shown in ***Fig. 1A***, high concentrations of MMA (> 120 mmol/L) significantly decreased the cell survival rate. Next, we determined the type of cell death induced by MMA at high concentrations by flow cytometry using Annexin V-FITC /PI and fluorescence staining. We found that exposure to 150 mmol/L MMA for 2 hours significantly increased the number of necrotic cells (Annexin V-FITC$^-$/PI$^+$) compared with control (***Fig. 1B*** and ***C***). However, the number of apoptotic cells induced by 150 mmol/L MMA (Annexin V-FITC$^+$/PI$^+$ or Annexin V-FITC$^+$/PI$^-$) is low, as the same in control group (***Fig. 1B***). Furthermore, we found that PEDF markedly decreased MMA-induced necrosis (***Fig. 1B*** and ***C***).

Considering the high concentration of MMA (> 120 mmol/L) has not been fully confirmed clinically, we further examined the chronic effects of MMA at low concentration on cell survival rate. As shown in ***Fig. 2A***, the chronic treatment of 30-90 mmol/L MMA for 24 also significantly decreased cell survival rate in H9c2 cells. Flow cytometry analysis and fluorescence staining data demonstrated that exposure to 75 mmol/L MMA for 24 hours significantly increased the number of apoptotic cells (Annexin V-FITC$^+$/PI$^+$ or Annexin V-FITC$^+$/PI$^-$) compared with controls (***Fig. 2B*** and ***C***). However, there was no change in the number of necrotic cells after exposure to 75 mmol/L MMA, compared with the control group (***Fig. 2B***). Importantly, we found that PEDF could markedly decrease MMA-induced apoptosis (***Fig. 2B*** and ***C***).

PEDF suppressed MMA-induced oxidative stress in H9c2 cells

To investigate whether the antioxidant activity is part

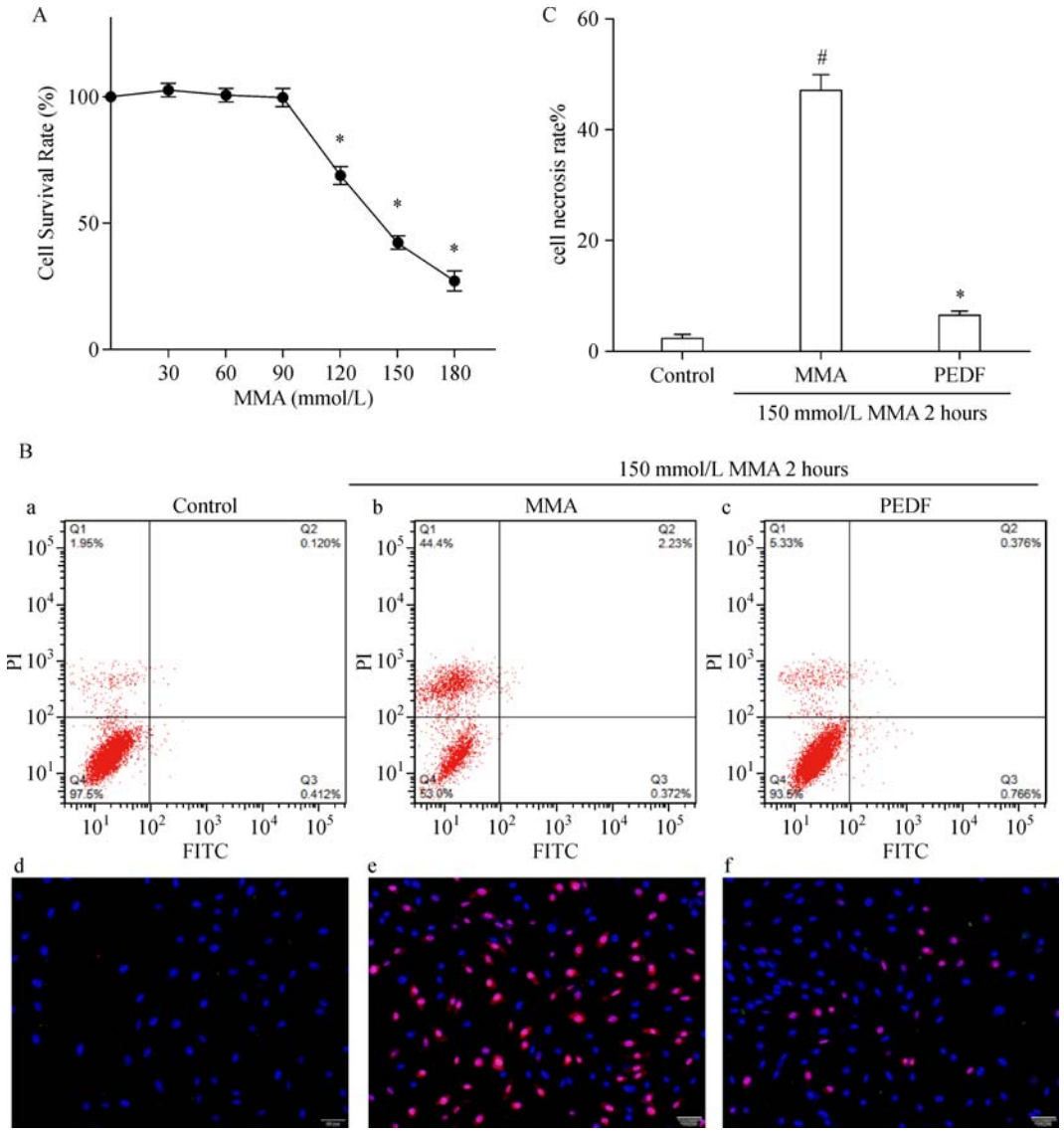

Fig. 1 **PEDF attenuates necrosis of H9c2 cells induced by high concentrations of MMA.** A: H9c2 cells were treated with different concentrations of MMA (0-180 mmol/L) for 2 hours. Then, the cell survival rate was examined by CCK-8 assay. Values are mean±SEM ($n = 3$). *$P < 0.05$ versus to the 0 mmol/L MMA group. B: Flow cytometrical analysis results (A–C) and fluorescence images (D–F) of high concentrations of MMA-induced cell death in H9c2 cells with or without PEDF are shown. Green is FITC while red is propidium iodide (PI). C: A statistical graph of annexin V-FITC/PI staining for high concentrations of MMA-induced necrosis in H9c2 cells with or without PEDF is shown. The averages were calculated using results from three independent experiments. Values are mean±SEM ($n = 3$). *$P < 0.05$ versus the MMA group. $^{\#}P < 0.05$ versus the control group. Bar = 50 μm.

of the mechanisms by which PEDF attenuated MMA-induced cytotoxicity in H9c2 cells, we first used fluorescent probes DCFH-DA to detect the effect of PEDF on ROS production in H9c2 cells. As shown in ***Fig. 3A*** and ***3C***, we found that high concentrations of MMA could produce high levels of ROS. Similarly, there were also high levels of ROS production after low concentrations of MMA treatment (***Fig. 3B*** and ***3D***). Pretreatment with PEDF for half an hour significantly inhibited ROS production (***Fig. 3A–D***). To further verify the effects of PEDF on MMA-induced oxidative stress, MDA levels and T-SOD activity were examined by special detection kits. As shown in ***Fig. 3E*** and ***3F***, PEDF could attenuate MMA-induced upregulation of MDA levels. In addition, PEDF could restore the downregulated levels of T-SOD activity induced by MMA. These data suggest that PEDF suppress MMA-induced oxidative stress in H9c2 cells.

PEDF blocked MMA-induced apoptosis via regulating the Akt and/or PEDF receptor-LPA-PPARγ pathway

To gain further insight into the mechanism by which PEDF attenuated MMA-induced apoptosis in H9c2

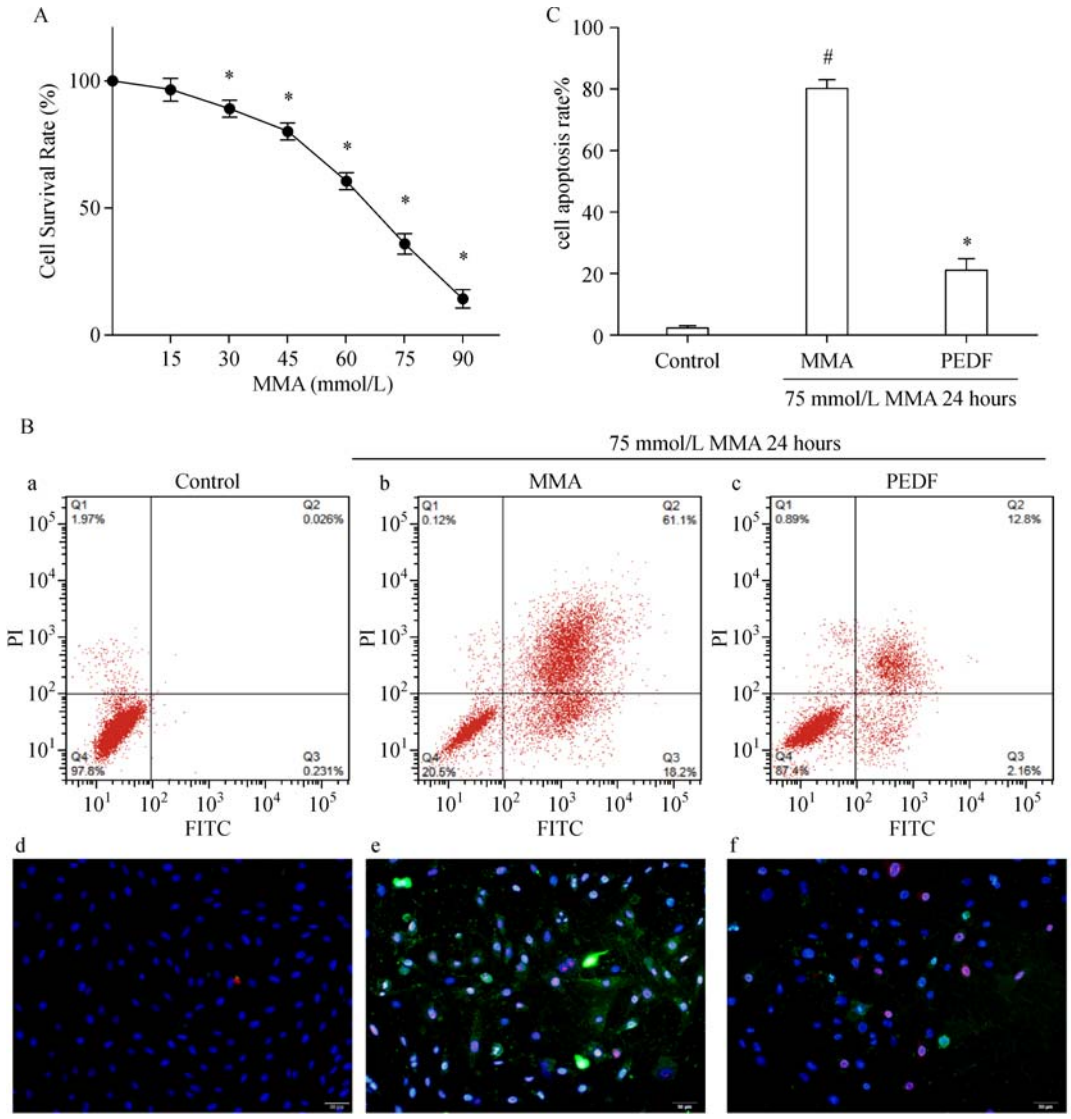

Fig. 2 **PEDF attenuates low concentrations of MMA-induced apoptosis in H9c2 cells.** A: H9c2 cells were treated with different concentrations of MMA (0-90 mmol/L) for 24 hours. Cell survival rate was examined by CCK-8 assay. Values are mean±SEM ($n = 3$). *$P < 0.05$ versus to the 0 mmol/L MMA group. B: Flow cytometrical analysis results (A–C) and fluorescence images (D–F) of low concentrations of MMA-induced cell death in H9c2 cells with or without PEDF are shown. Green is FITC while red is PI. C: A statistical graph of annexin V-FITC/PI staining for low concentrations of MMA-induced apoptosis in H9c2 cells with or without PEDF is shown. The data averages were calculated using results from three independent experiments. Values are mean±SEM ($n = 3$). *$P < 0.05$ versus the MMA group. $^{\#}P < 0.05$ versus the control group. Bar = 50 μm.

cells, we examined the levels of cleaved caspase-3 and Akt phosphorylation by Western blot analysis. As shown in ***Fig. 4A***, 75 mmol/L MMA could significantly increase cleaved caspase-3 levels, further demonstrating that MMA induced apoptosis in H9c2 cells. In addition, pretreatment with PEDF could suppress MMA-induced upregulation of cleaved caspase-3. We also found that MMA-induced downregulation of phosphorylated Akt level was significantly inhibited by PEDF. It has been reported that PEDF promotes cell survival by binding to and stimulating PEDF receptor, subsequently inducing phospholipase A2 liberating fatty acids and lysophosphatidic acid (LPA) from phospholipids. LPA could act as a second messeger to activate the PPARγ pathway. To investigate whether the PEDF receptor-LPA-PPARγ pathway was involved in the protective effect of PEDF, PEDF receptor inhibitor Atglistatin was used in the present study. As shown in ***Fig. 4C*** and ***D***, PEDF could enhance the levels of PPARγ and LPA. Furthermore, PEDF-induced upregulation of PPARγ and LPA levels was suppressed by Atglistatin. These data suggested that PEDF could suppress MMA-induced apoptosis through regulating the Akt or/and PEDF-LPA-PPARγ pathways.

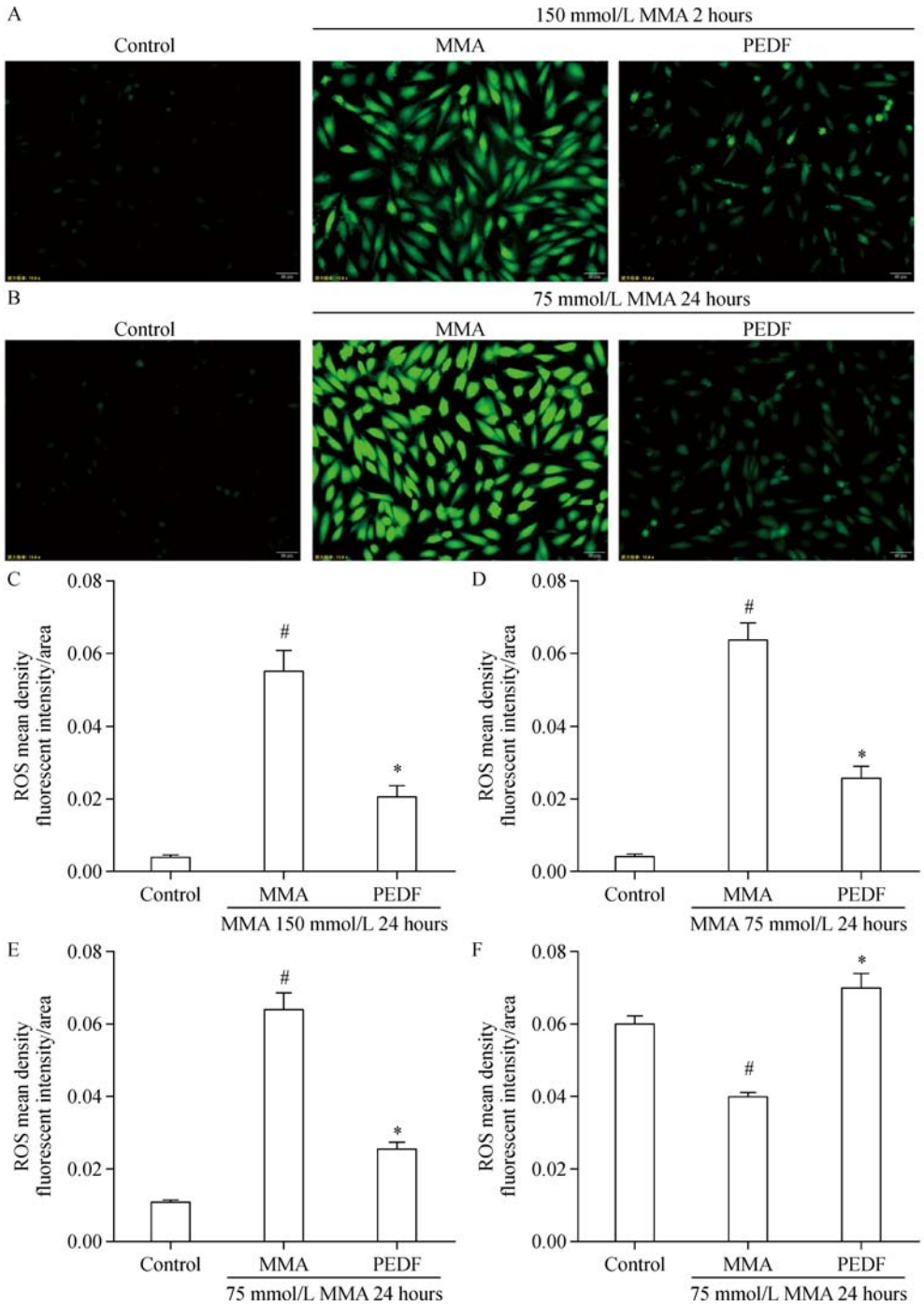

Fig. 3 **PEDF suppresses MMA-induced oxidative stress in H9c2 cells.** A-D: H9c2 cells were pretreated with or without PEDF, and then incubated with 150 mmol/L MMA for 2 hours (A and C), or 75 mmol/L MMA for 24 hours (B and D). The intracellular ROS levels were measured by DCFH-DA fluorescence probe. E: PEDF inhibited MMA-induced upregulation of MDA levels. F: PEDF increased intracellular T-SOD activity. Values are mean±SEM (n = 3). *P < 0.05 versus the MMA group. $^{\#}P$ < 0.05 versus the control group. Bar = 50 μm.

Discussion

Since bone cement was first used in clinics in 1970s, cardiac arrest cases have been reported following the use of MMA[17]. Although new types of bone cement such as calcium phosphate cement or calcium sulfate cement were invented, their low fracture toughness and poor mechanical reliability restrict their application[18–19]. Acrylic bone cement is still irreplaceable in clinics now. With the increased use of acrylic bone cement during current orthopedic implant treatment, the number of BCIS cases is on rise[20]. However, the pathophysiology of BCIS is not entirely clear. Studies have reported that bone cements have toxicity on the body, including the myocardium. Yasuda *et al*. first reported that MMA monomer caused a dose-dependent

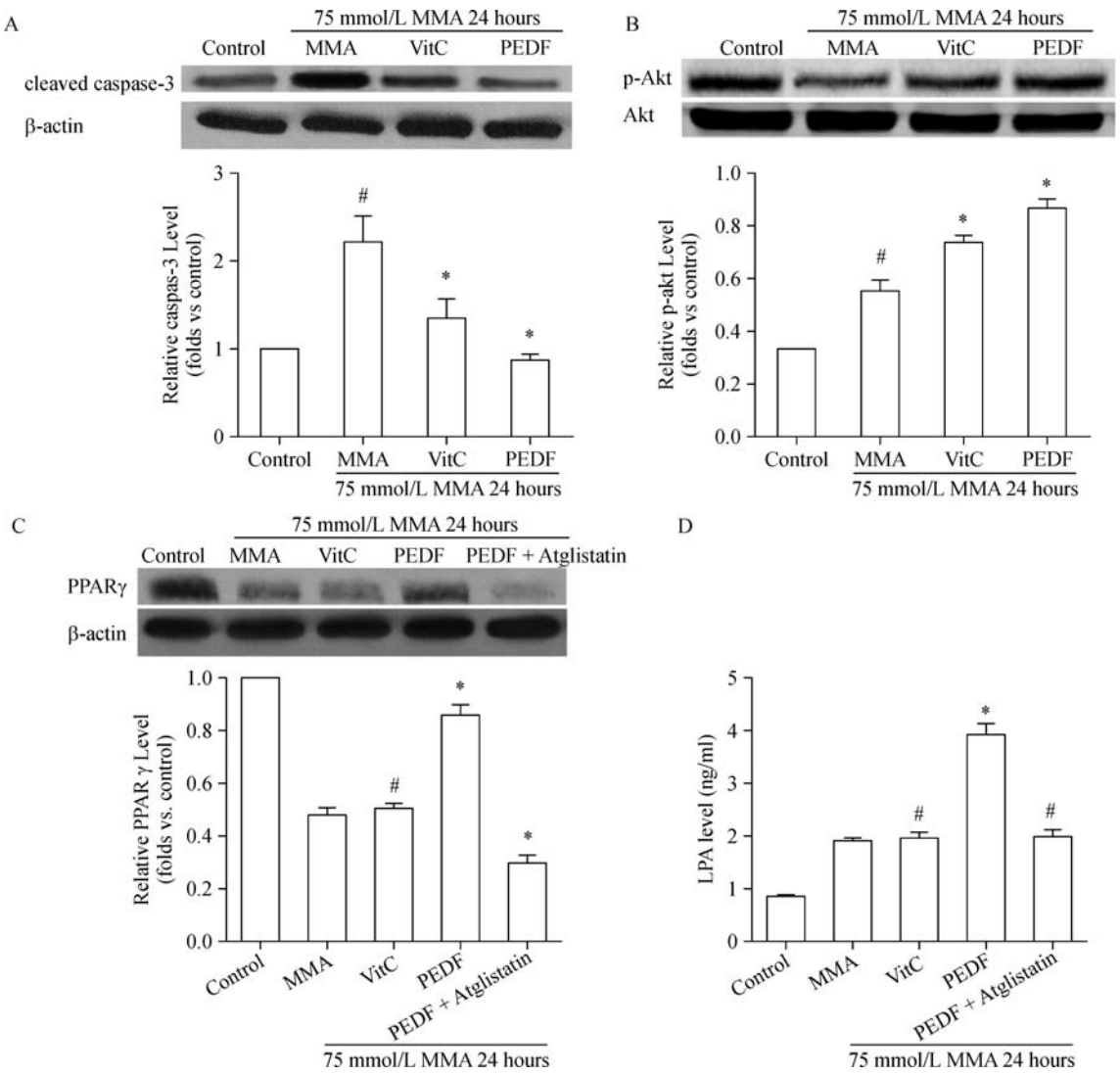

Fig. 4 **PEDF blocks MMA-induced apoptosis via regulating Akt activities in H9c2 cells.** H9c2 cells were pretreated with or without PEDF, and then incubated with 75 mmol/L MMA for 24 hours. The levels of cleaved caspase-3 (A), Akt phosphorylation (B) and PPARγ (C) were examined by Western blotting assays. The level of lysophosphatidic acid (LPA) was determined by ELISA (D). Quantification is shown in lower panels. Values are mean±SEM (n = 3). *P < 0.05 versus the MMA group. #P < 0.05 versus the control group.

decrease in the maximum velocity of shortening and the maximum force development of dog myocardium[13]. Kim *et al*. further demonstrated that MMA monomer depressed Ca^{2+} influx through cardiac membrane and depolarization-activated sarcoplasmic reticulum Ca^{2+} released through whole-cell voltage clamp measurements[21]. Furthermore, studies found that the cytotoxicity of acrylic bone cement is partly attributed to ROS formation, leading to cell death[22]. Consistent with these studies, our present study also found that MMA stimulation could produce high levels of ROS and decrease cell survival rate in H9c2 cells. The mode of cell death by MMA is related to drug concentration, with necrosis occurring with high concentrations and apoptosis with lower concentration and long time treatment. These data suggest that the occurrence of BCIS might be partly due to the cytotoxicity of MMA on cardiomyocytes.

PEDF-R is a transmembrane protein with phospholipase A2 and triglyceride lipase activity[23]. When PEDF binds to and stimulates PEDF receptor (PEDFR), LPA can be liberated from phospholipids to act as signal transducer. It has been reported that LPA can act on PPARγ[24]. The activation of PPARγ reduces ROS production and suppresses apoptosis and necroptosis[25]. It has been reported that PPARs is a ligand activated transcription factor, which binds to specific DNA response element and then controls the gene expression of antioxidative enzymes[26]. Our previous studies have demonstrated that PEDF attenuates hypoxia-induced apoptosis and necrosis in H9c2 cells via PEDF receptor[27]. In the present study, PEDF was

shown to upregulate the levels of LPA and PPARγ, and enhance SOD activity. These data suggest that PEDF might protect against MMA-induced cytotoxicity partly through PEDFR-LPA-PPARγ pathway.

The serine-threonine kinase Akt is a non-receptor kinase that plays critical roles in cell growth, survival and metabolism[28]. PEDF could increase SOD activity and phosphorylation of Akt to prevent endothelial dysfunction induced by acute ethanol intake. Consistent with these studies, our present study also demonstrated that PEDF could attenuate MMA-induced cytotoxicity and increase SOD activity and Akt phosphorylation. There are also studies indicating that LPA could activate the PI3K/Akt pathway to exert protective effects[29]. Our present study found that PEDF could increase LPA levels and enhance Akt phosphorylation in H9c2 cells[30]. These data suggest that the activation of PI3K/Akt pathway is also involved in the protective effects of PEDF against MMA-induce cytotoxicity.

In conclusion, the present results showed that MMA induced apoptosis at lower doses and necrosis at higher doses in H9c2 cells. Pretreatment of PEDF could provide protection against MMA-induced cytotoxicity. These effects were attributed to the attenuation of MMA-induced oxidative stress and increased Akt activity. These findings suggest that PEDF and vitamin C may be beneficial in improving the therapeutic strategy of BCIS.

Acknowledgments

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